

Studies on  
ALBUMIN AND ACUTE PHASE REACTANTS  
IN DIFFERENT BODY FLUIDS

with special reference to the immediate  
and slow bromcresol green reaction

by

Jan E.C. Gustafsson

*Hund*

*1981.*



**DEPARTMENT  
OF  
TECHNICAL ANALYTICAL  
CHEMISTRY**



**CHEMICAL CENTER  
LUND INSTITUTE OF TECHNOLOGY  
LUND—SWEDEN**



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Studies on albumin and acute phase reactants in different body fluids with special reference to the immediate and slow bromcresol green reaction.

Referat (sammandrag)

The reaction between bromcresol green and different body fluids, e.g. serum, urine and CSF, has been studied. It was found early on that the reaction between BCG and serum proceeds in two steps; albumin causes the immediate reaction while other proteins cause the slow reaction (haptoglobin, transferrin and  $\alpha_1$ -antichymotrypsin)

The measure of the slow BCG reaction is defined as the difference between the "albumin" concentration obtained after 60 min and 0 min reaction time. This is called  $\Delta C$  and is expressed in g/liter. It was shown that  $\Delta C$  was a measure of the acute phase reactants in serum.

Erythrocyte sedimentation rate (ESR) was compared with results from the immediate and slow BCG reactions. The most useful information was obtained by combining the BCG method with a total protein determination. In a diagram  $\Delta C$  (Y-axis) was compared with the difference (x-axis) between total protein and albumin values. For the patients in this study a complete separation between normal ESR:s and pathological ESR:s was obtained.

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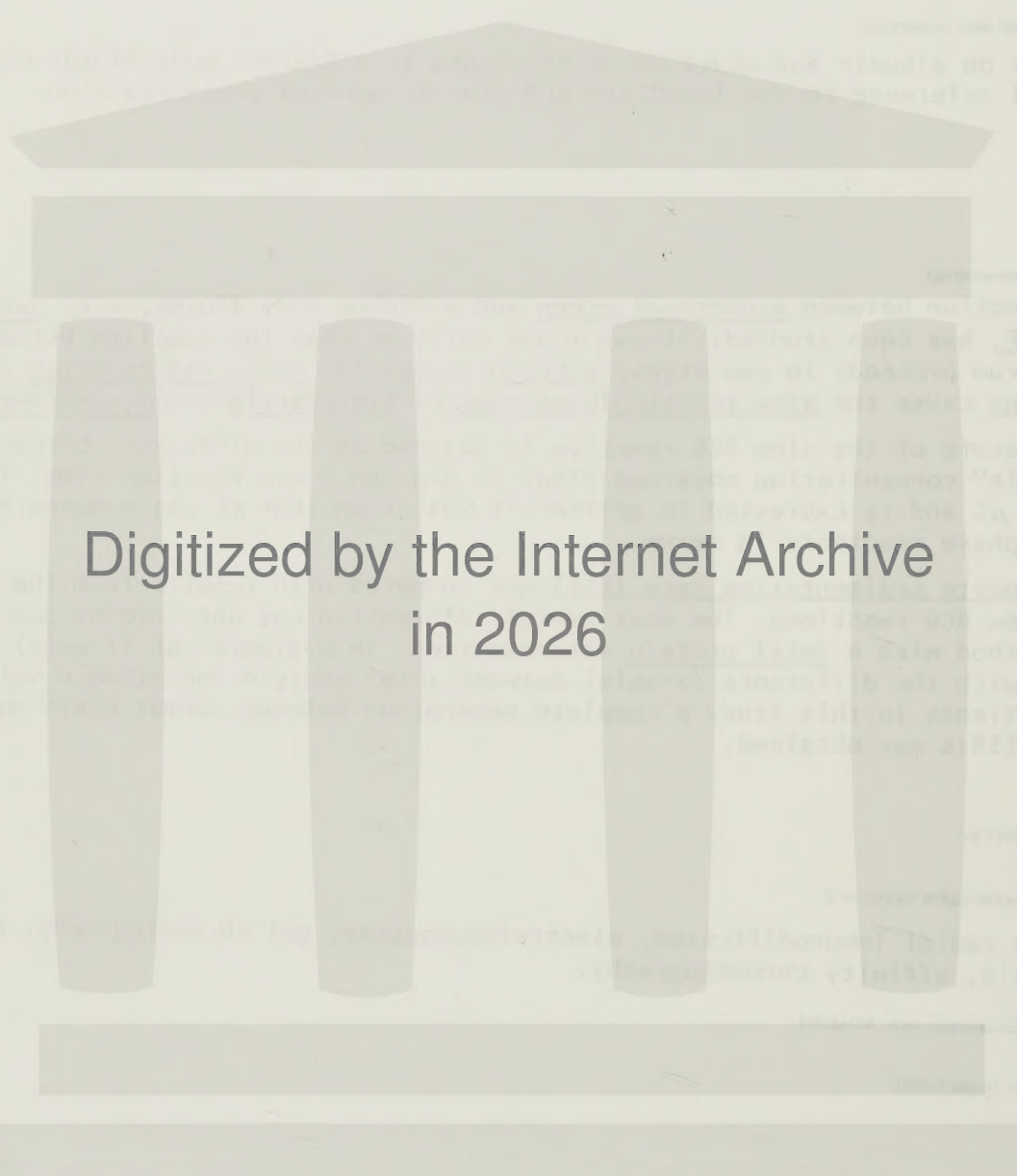
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and slow bromcresol green reaction

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Jan E.C. Gustafsson

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This thesis is mainly based on the following papers, referred to in the text by their Roman numerals:

- I Gustafsson, J.E.C.  
Improved Specificity of Serum Albumin Determination and Estimation of "Acute Phase Reactants" by Use of the Bromcresol Green Reaction. Clin. Chem. 22, 616-622 (1976).
- II Gustafsson, J.E.C. and Uzqueda, H.R.B.  
Urinary Albumin Determination by the Immediate Bromcresol Green Method. Clin. Chim. Acta 90, 241-248 (1978).
- III Gustafsson, J.E.C. and Uzqueda, H.R.B.  
The Influence of Citrate and Phosphate on the Mancini Single Radial Immunodiffusion Technique and Suggested Improvements for the Determination of Urinary Albumin. Clin. Chim. Acta 90, 249-257 (1978).
- IV Gustafsson, J.E.C.  
Automated Serum Albumin Determination by Use of the Immediate Reaction with Bromcresol Green Reagent. Clin. Chem. 24, 369-373 (1978).
- V Gustafsson, J.E.C. and Uzqueda, H.R.B.  
Albumin Determination in Cerebrospinal Fluid by a Manual and an Automated Immediate Bromcresol Green Method. Submitted for publication.
- VI Gustafsson, J.E.C. and Uzqueda, H.R.B.  
Simplified Procedure for Urinary Albumin Determination by the Immediate Bromcresol Green Method. Submitted for publication.
- VII Gustafsson, J.E.C.  
Identification of Haptoglobin, Transferrin and  $\alpha_1$ -Antichymotrypsin to Cause the Slow Bromcresol Green Reaction with Human Serum, and further Demonstration of the Specificity for Albumin of the Immediate Reaction. Submitted for publication.



ABBREVIATIONS

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To my family

ABBREVIATIONS

|            |                                                                                                                                           |
|------------|-------------------------------------------------------------------------------------------------------------------------------------------|
| Achy       | $\alpha_1$ -antichymotrypsin                                                                                                              |
| APR        | acute phase reactant                                                                                                                      |
| APRs       | acute phase reactants                                                                                                                     |
| BCG        | bromcresol green                                                                                                                          |
| BCP        | bromcresol purple                                                                                                                         |
| $\Delta C$ | the difference between the "albumin" concentration obtained after 60 min and 0 min reaction time by the BCG method (expressed in g/liter) |
| CA         | cellulose acetate                                                                                                                         |
| CRP        | c-reactive protein                                                                                                                        |
| CSF        | cerebrospinal fluid                                                                                                                       |
| EIA        | electroimmunoassay (Laurell "rocket" technique)                                                                                           |
| ESR        | erythrocyte sedimentation rate                                                                                                            |
| HABA       | 2-(4'-hydroxyazobenzene)-benzoic acid                                                                                                     |
| Hb         | hemoglobin                                                                                                                                |
| HbBC       | hemoglobin binding capacity (for Hp determination)                                                                                        |
| Hp         | haptoglobin                                                                                                                               |
| Ig         | immunoglobulin (i.e. IgA, IgD, IgE, IgG, and IgM)                                                                                         |
| MO         | methyl orange                                                                                                                             |
| SRID       | single radial immunodiffusion (Mancini technique)                                                                                         |
| Tf         | transferrin                                                                                                                               |
| TIBC       | total iron binding capacity                                                                                                               |
| TP         | total protein                                                                                                                             |



## C O N T E N T S

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## INTRODUCTION

The field of protein chemistry and its use in biology and medicine has expanded greatly in recent years. The development of methods, such as electrophoresis, chromatography and immunochemistry, capable of identifying and quantitating individual proteins rather than complex mixtures of proteins was a very important step forward in this discipline.

Human blood plasma is a complex solution containing hundreds of different proteins, some of them in very low concentrations. The biological functions of the individual proteins vary. They may act as enzymes, enzyme inhibitors, hormones, coagulation and fibrinolysis factors, transport proteins, complement components or immunoglobulins (antibodies). The approximate concentration range of some human plasma proteins is shown in Table I.

Table I. The concentrations of some human plasma proteins divided into four concentration groups. (Proteins within parentheses, present in plasma only).

| Conc. range<br>> 10 g/l | Conc. range<br>1-10 g/l       | Conc. range<br>0.1-1 g/l           | Conc. range<br>< 0.1 g/l     |
|-------------------------|-------------------------------|------------------------------------|------------------------------|
| Albumin                 | $\alpha_1$ -lipoprotein (HDL) | Prealbumin                         | Prothrombin                  |
| IgG                     | $\alpha_1$ -antitrypsin       | Orosomucoid                        | Transcortin                  |
|                         | Haptoglobin                   | $\alpha_1$ -B-glycoprotein         | TBG                          |
|                         | $\alpha_2$ -macroglobulin     | $\alpha_1$ -antichymotrypsin       | $\alpha_1$ T-glycoprotein    |
|                         | $\beta$ -lipoprotein (LDL)    | Gc-globulin                        | Retinolbinding protein       |
|                         | Transferrin                   | Inter- $\alpha$ -trypsin inhibitor | Zn- $\alpha_2$ -glycoprotein |
|                         | (Fibrinogen)                  | Antithrombin III                   | C1s-component                |
|                         | IgA                           | Ceruloplasmin                      | Serum-cholinesterase         |
|                         | IgM                           | $\alpha_2$ HS-glycoprotein         | C2-component                 |
|                         |                               | C1s-inactivator (Plasminogen)      | C5-component                 |
|                         |                               | Cold-insoluble $\beta$ -globulin   | CRP                          |
|                         |                               | Haemopexin                         | $\beta_2$ -microglobulin     |
|                         |                               | C1r-component                      | IgD                          |
|                         |                               | C3-component                       | IgE                          |
|                         |                               | C4-component                       | Other enzymes                |
|                         |                               | $\beta_2$ -glycoprotein I          | Other hormones               |
|                         |                               | C3-proactivator                    |                              |
|                         |                               | C1q-component                      |                              |
|                         |                               | C8-component                       |                              |

Several comprehensive monographs on human plasma proteins have been published, covering not only the protein chemistry but also some biological and medical applications. These include Schultze and Heremans (1), Blombäck and Hanson (2), Putnam (3), Ritzmann and Daniels (4), And Bianchi et al. (5).

### Albumin, General

Several hundred papers have been written on serum albumin. There are also many comprehensive reviews. Pertinent reviews are those of Hughes (6), Foster (7), Putnam (8), Watson (9), Schultze and Heremans (1), Peters (10), Rotschild et al. (11,12), Andersson (13) and Peters (14).

Human serum albumin is the most abundant (comprising about half the protein content) and the most studied of the proteins of the circulation. The ease of its isolation and its high stability and solubility have made it a prototype for the study of protein chemistry. Its name derives from the early name for proteins, albumen, derived from the Latin word albus, white (white of an egg). Albumins in serum were initially defined as those proteins which are soluble in water in the absence of salts, in contrast to globulins, which are insoluble under such conditions.

Three main functions of albumin have been recognized; its osmotic effect (about 80% of the total in blood), its function as a reserve protein and as a transport protein. It is still unclear if it has other functions as well. As a transport protein, albumin is known to bind several different types of compounds, anionic, cationic as well as nonionic, (i.e. free fatty acids with long carbon chains, bilirubin, hemins, calcium, copper, hormones and catecholamines). Various drug substances are also transported by albumin. This area is currently subject to intensive studies because of its great pharmacological importance. However it is important to know that all these functions of albumin can be substituted by other proteins in the organism. The most striking example is patients with an-albuminemia, who in the almost complete absence of circulating albumin, remain edema-free (1).

The work on the important question of the connection between structure and function has only just started and little data is yet available.



The complete amino acid sequence of human serum albumin was recently published independently by Behrens et al. (15) and Meloun et al. (16). The albumin molecule is a single peptide chain of more than 580 amino acid residues. The covalent structure for albumin is pictured as a series of double loops formed by disulphide bonds. This pattern suggests that the molecule is arranged naturally into three "domains". Andersson (13) has also constructed a model containing three "domains" and shows how the hydrophilic, hydrophobic and intermediary amino acid units are arranged in the molecule.

Human albumin is known to be synthesised by the liver, but it is still unclear where its catabolism occurs. It pervades almost every fluid or secretion of the body to some extent (1). The normal level of albumin in human serum is 42 g/liter, with a range of 35-50 g/liter (17).

Diseases affecting the level of serum albumin almost invariably cause hypoalbuminemia. Hyperalbuminemia can be caused by either dehydration, an overshoot of the regulatory mechanism, or laboratory errors. Hypoalbuminemia results from four main causes; reduced synthesis, increased catabolism, abnormal losses and pathological distribution. A decrease in the albumin level is found during various pathological conditions. The most severe hypoalbuminemia is found in nephrotic syndrome or protein-losing enteropathy. Hepatic cirrhosis, glomerulonephritis, malnutrition, malignancy and other diseases decrease the serum level of albumin. The inflammatory reactions are also associated with reduced levels of albumin besides the increase in acute phase reactants (see below).

#### Albumin, Determination in Serum, Urine and CSF

The clinical interest in the determination of the albumin content in different body fluids increases steadily. Total protein determinations are often preferably substituted by determinations of albumin, since the albumin content is more closely correlated to the course of most diseases than the total protein content of plasma. Moreover it is difficult to find suitable standards for the total protein methods, as all these methods exhibit different sensitivities towards the different proteins. Furthermore the protein pattern varies considerably between health and

different diseases. The general trend in clinical chemistry is to substitute general proteins methods by methods which determine specific proteins.

The clinical uses of serum albumin determinations are numerous and well known. To the physician a normal albumin level can indicate a state of well-being, in contrast to the many disease states which are accompanied by a fall in albumin and a rise in globulin levels.

Albumin determination in urine is used to establish a proteinuria (albuminuria), and the degree of it (i.e. as albumin clearance). Albumin determinations in urine may be useful for patients with pregnancy toxico-sis (hypertoni during pregnancy).

Increased protein content in the cerebrospinal fluid (CSF) usually reflects an increased passage of plasma proteins through a damaged blood-CSF barrier, or a liberation of proteins from the central nervous system. The damaged barrier gives an increase in the CSF/plasma ratio of albumin. Local immunoglobulin G (IgG) production is found by either comparing the CSF/plasma ratios for albumin and IgG graphically (18) or calculating an IgG/albumin index, i.e. the quotient of the CSF/serum ratios of IgG and albumin (19).

Many methods have been described for determining albumin in serum. They can be divided into four main groups, each related to a specific property of albumin: solubility, electrophoretic mobility, reaction with specific antibodies and binding of dyes.

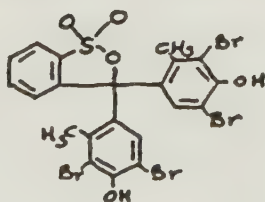
The high solubility of albumin in concentrated salt solutions and in acid-alcohol solution facilitates its separation from globulins. Once purified, albumin can be measured by any total protein method or by turbidity determination. Watson (20) and Majoor (21) used 1.8 mol/liter sodium sulphate while Wolfson et al. (22) used 2.4 mol/liter sodium sulphite. Acid-alcohol methods use trichloroacetic acid (20,23,24) perchloric acid (25), or hydrochloric acid (26). Although difficult to automate, a precipitation technique measuring turbidity in a 1.8 mol/liter sodium sulphate was developed by Glenn (27). Saifer and Marven (28) used the tryptophan reaction.

The first electrophoretic technique used for separation and quantitation of plasma proteins was the moving boundary technique of Tiselius. This was later replaced by zone electrophoresis on paper then by cellulose acetate (CA) electrophoresis (29-32). These techniques have often been used as a reference for other procedures, despite the fact that different proteins show different staining intensity with the dyes used. Electrophoretic techniques followed by staining are also difficult to automate.

Immunological techniques used for albumin determination include the Mancini single radial immunodiffusion (SRID) (33), Laurell "rocket" technique or electro immuno assay (EIA) (34) and precipitation techniques quantitated by the turbidity of suspension (35,36) or nephelometric assay also with polymer enhancement (37). Although specific and reliable, SRID and EIA cannot be automated, rendering them expensive (reagent and labour costs). The two precipitation techniques are often automated, but reagents are still expensive. A radio-affinity assay (38) and a thermometric enzyme linked immunosorbent assay (39) have also been presented.

The fact that albumin binds various dyes has been used for its quantitation. Many dyes have been used, e.g. 2-(4'-hydroxyazobenzene)-benzoic acid (HABA) (40-42), methyl orange (MO) (43,44), bromcresol green (BCG) (see below) and bromcresol purple (BCP) (45-49). The HABA and MO methods show interference with bilirubin and certain drugs and are now substituted by BCG or BCP. BCG and BCP have been claimed not to be fully specific for albumin.

The BCG method is based on the "protein error" in acid-base titrations when certain proteins are present. The Kekulé structural formula for BCG is:



The BCG method is currently the most widespread of all albumin methods. According to Peters (14) 48% of 1160 U.S. laboratories surveyed in 1974 used BCG, while 30% used HABA. Thus at least 78% used the dye binding methods. In Sweden at least 80-90% currently use the BCG method, in Upp-



sala and Örebro regions 100%. 53 out of 84 laboratories in Scandinavia use the BCG method according to Landaas et al. (50).

Since the work of Rodkey (51,52) who used a neutral buffered solution (giving high blank values), a large number of papers have been presented on the BCG method used at a low pH 3.8-4.4 (e.g. 53-81 and paper I-VII). Although its specificity is better than the HABA method (54), it has earlier only been recommended as a screening method (55-57). Results by the BCG method did not compare well with more specific immunological methods for albumin determination, especially in the clinically important lower concentration range (56,58,I), the BCG method giving artifactually too high values for such sera.

The work on this thesis was started due to the large differences in albumin results between the laboratories participating in a regional control programme (Figure 1). By sending out reference albumin samples in addition to the regular samples, I was able to recalculate all the results on the basis of the values found for these reference samples in order to minimize the standardization error. A certain improvement could then be seen, but the main error remained. I then started the methodological work presented in this thesis. The result was the improved immediate BCG method presented in paper I. This has proved to be a specific and reliable method as has been confirmed by several other authors (see below). Further work made this method useful also for albumin determinations in urine and CSF. Automated procedures were also developed.

### Acute Phase Reactants

There are many indicators of inflammatory disease. Those most relied upon to define the active state of inflammation are fever, leucocytosis, increased erythrocyte sedimentation rate (ESR) and increase in the level of acute phase reactants (APRs). APRs will be discussed in some detail below as the slow reaction between serum samples and BCG will give a measure of APRs. ESR will also be discussed in this thesis.

Some plasma proteins increase while others decrease in concentration in acute inflammatory conditions (e.g. after trauma or during acute infections) as can be seen in Table II. The former are termed "acute phase proteins" or "acute phase reactants".

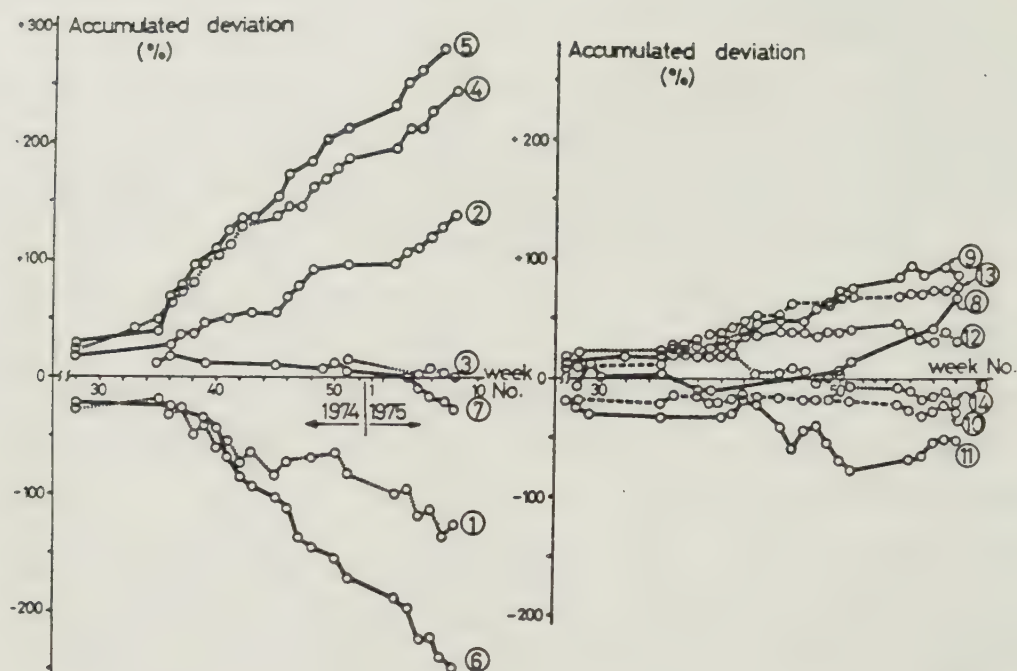


Figure 1. Albumin results from the regional control programme, shown as cumulative diagrammes (June 1974-March 1975). 14 different laboratories participate (①-⑭). They are randomly put in the left or right part of the figure.

Pertinent reviews on APRs are included in Ritzmann & Daniels (4), Bianchi et al. (5), Blombäck & Hanson (2) and Laurell (82). The level of APRs is a valuable tool in the detection, diagnosis, prognosis and therapeutic monitoring of diseases involving tissue damage and inflammation.

Transferrin (Tf) shows an increased synthesis rate during an inflammatory reaction, but its catabolism is increased in excess, resulting in a decreased plasma concentration (5). Thus Tf is generally not included among APRs.

APRs can be divided into three main groups as regards their rate of increase in concentration after the onset of the inflammatory reaction. The first group consists of C-reactive protein (CRP) and  $\alpha_1$ -antichymotrypsin (Achy), which increase rapidly within a few hours. The second

Table II. Plasma protein changes during an acute inflammatory reaction.  
Compiled from (2,4,5,82).

| Increased plasma concentration      | Decreased plasma concentration |
|-------------------------------------|--------------------------------|
| C-reactive protein (CRP)            | Prealbumin                     |
| Orosomucoid                         | Albumin                        |
| $\alpha_1$ -antichymotrypsin (Achy) | Transferrin (Tr)               |
| Haptoglobin (Hp)                    | $\alpha$ -lipoprotein (HDL)    |
| $\alpha_1$ -antitrypsin             |                                |
| Fibrinogen                          |                                |
| Ceruloplasmin                       |                                |
| C3-component                        |                                |
| C4-component                        |                                |
| Hemopexin                           |                                |
| Prothrombin                         |                                |

group, consisting of fibrinogen, haptoglobin (Hp), orosomucoid and  $\alpha_1$ -antitrypsin, shows a significant increase in plasma levels with a maximum after about one week, and a return to normal levels within 4-6 weeks. The remaining proteins shown in Table II form the third group which shows a slow increase with maximal levels after one to two weeks, and a slow return to normal levels. The increase is not so pronounced as for the proteins in groups 1 and 2.

The most reliable indicators of inflammatory processes are orosomucoid, Achy and CRP (2). Other APRs are not completely reliable due to complicating factors. Increased catabolism or elimination can reduce the plasma levels. Intravascular coagulation or fibrinolysis decreases the fibrinogen level, while the haptoglobin level decreases due to increased haptoglobin consumption, e.g. by hemolysis or increased ineffective erythropoies. Complement activation can result in an unchanged or a decreased plasma level of C3 and C4 components. As regards  $\alpha_1$ -antitrypsin, increased catabolism may occur due to increased leucocyte damage.

The mechanisms of the acute phase response have been subject to considerable debate. It has been demonstrated that APRs are produced in the liver and released into the hepatic circulation (4). It was also shown that the increase in APRs is the result of an increased synthesis rate. The exact details are however little understood. The APRs rise after tissue necrosis and inflammation, but additional factors modify their kinetics. Regulation by hormones such as adrenal steroids, insulin, growth hormone, and thyreotropin must be considered. Some data suggest



that disruption of the cellular lysosomes is an early step in a series of events leading to the eventual stimulation of acute phase protein synthesis (4). Additionally the possible effects of prostaglandins and endotoxins must be considered.

The rise in APRs which follow many disease states merely indicates the presence of an inflammatory reaction, without providing any details of the type or localization, although characteristic changes in the plasma protein pattern are obtained e.g. in liver diseases. The clinical primary diagnostic use of plasma protein determinations is thus somewhat limited, but important information of the level and/or intensity can be obtained. Single or widely-spaced determinations of APRs are of limited help. The intensity of the inflammatory process should rather be followed by serial determinations. This adds important diagnostic and prognostic information which increases the usefulness of the determinations.

### Haptoglobin

Hp was discovered by Polonovski and Jayle in 1939 (83). Comprehensive reviews are those of Putnam (3), Schultze & Heremans (1), Blombäck & Hanson (2) and Salmi (84).

The biological function of Hp is to bind free hemoglobin in plasma in a one-to-one ratio in a relatively high molecular weight complex. This prevents iron losses through urinary excretion and protects the kidneys from damage by hemoglobin. The Hp-Hb-complex is eliminated from the blood plasma and metabolised in the reticuloendothelial system. Hp also acts as an APR (see above).

Smithies (85) demonstrated that there are three major Hp types. They are now designated as Hp 1-1, Hp 2-1, and Hp 2-2. Hp 1-1 is a monomer whereas the other two are polymers. Hp consists of  $\alpha$ - and  $\beta$ -chains. The  $\beta$ -chain is identical in all three phenotypes while the  $\alpha$ -chain type is either 1 or 2. (Type 1 can be further divided into S and F). Perhaps the difference in the alpha chains explains why Hp 2-1 and Hp 2-2 tend to polymerize and Hp 1-1 stays as a monomer.

Hp is an  $\alpha_2$ -globulin. The molecular weight of Hp 1-1 is 100 000 (1), and the isoelectric point (pI) is pH 4.2-4.25 while pI is 4.1-4.2 for Hp 2-2. The average molecular weight is about 220 000 for Hp 2-1 and about 400 000 for Hp 2-2 (3). Hp is easily denaturated especially at acidic pH. Acidification of whole serum to pH 4.2 (compare the reaction conditions in the BCG method below) denatures 80% of the Hp in 30 min (3).

Anhaptoglobinemia is common in the newborn (cord blood and first days), rising to adult levels in the first year (84).

Hp is present in low concentrations in many extravascular physiological fluids including CSF and urine.

In general, serum Hp is decreased in hemolytic anemias and is increased in a variety of inflammatory conditions, upto 5 fold normal levels. Even higher levels are found in the nephrotic syndrome.

### Transferrin

Most of the acid soluble iron in plasma is reversibly bound to a specific metal-combining protein, which was termed transferrin by Holmberg & Laurell (86) for its function of transporting iron. Comprehensive reviews are those of Putnam (3), Blombäck & Hanson (2), Chrichton (87), Schultze & Heremans (1) and Miescher & Jaffé (88).

The biological function of Tf is to bind and transport iron.  $\text{Fe}^{3+}$  is very sparingly soluble in aqueous solutions. The solubility product of  $\text{Fe}(\text{OH})_3$ , is about  $10^{-36}$ , which gives the maximum concentration in biological fluids of less than  $10^{-14}$  mol/liter. The turnover of hemoglobin iron is about 30 mg, so the need for an iron transport system is clear. Tf fulfills this function.

Human Tf consists of a single polypeptide chain with a nonrepeating sequence (2,3). Each Tf molecule can combine with two atoms of ferric iron in an ionic bonding in which one bicarbonate ion is taken up per iron atom (2,3,87). A variety of other anions may substitute for carbonate under suitable experimental conditions (87).

Tf is a  $\beta$ -globulin, and comprises much of the  $\beta$ -globulin fraction. Its molecular weight is now estimated to be 77 000 (2,87,3), and the isoelectric point of apotransferrin is pH 5.8 and of fully saturated Tf, pH 5.45 (3). Electrophoresis below the isoelectric point produces an anomalous pattern (89). This may result from an isomerization reaction. Furthermore, the complex between Tf and  $\text{Fe}^{3+}$  is stable only in the pH range from 7.5 to 10, but begins to dissociate at pH 6.5 and is completely dissociated on acidification to pH 4 (3). Compare the reaction conditions in the BCG method (pH 4.2, with a surfactant). It has also been shown (3), that Tf containing iron is much more resistant to denaturation than apotransferrin.

The normal Tf content corresponds to an iron-binding capacity ranging between 2.5 to 4.0 mg/liter serum. This corresponds to 2.0 to 3.2 g/liter (mean 2.6) Tf in serum (3). Thus, the Tf is saturated to about one third in healthy subjects. In accounting for essentially all of the acid-soluble, protein-bound iron in plasma, Tf fulfills three functions in iron regulation: it governs the transport of free iron to the reticuloendothelial tissues, it acts as a buffer to prevent iron intoxication and to minimize changes in the activity of ferric iron in the plasma, and it prevents undue loss of iron by urinary excretion.

Many conditions affect the plasma Tf concentration. Normally minute amounts of Tf are lost in the urine, except in disease states with massive proteinuria. Factors known to increase the plasma concentration of Tf include iron deficiency, pregnancy, estrogen administration, cortisol administration and acute hypoxia. A decreased synthesis rate can occur in fasting, malnutrition, cirrhosis and possibly in iron overload conditions. Increased Tf catabolism has been found in infections, nephrosis and hemolytic anemias. Thus decreased levels are found in various inflammatory conditions. The decrease is then down to about 70% of normal Tf concentration (82).

#### $\alpha_1$ -Antichymotrypsin

The third protein, shown to react slowly with BCG (VII), is  $\alpha_1$ -antichymotrypsin (Achy). Achy is a protease inhibitor, chymotrypsin being



the inhibitory target. The major protease inhibitors are shown in Table III.

Table III. The major protease inhibitors in human plasma <sup>1</sup>.

| Inhibitor                          | Molecular weight | Normal plasma concentration (g/liter) | Inhibitory target <sup>2</sup> |   |   |   |   |
|------------------------------------|------------------|---------------------------------------|--------------------------------|---|---|---|---|
|                                    |                  |                                       | a                              | b | c | d | e |
| $\alpha_1$ -antitrypsin            | 54 000           | 2.0-4.0                               | +                              | + | + | - | - |
| $\alpha_1$ -antichymotrypsin       | 69 000           | 0.4-0.6                               | -                              | + | - | - | - |
| Inter- $\alpha$ -trypsin inhibitor | 160 000          | ~0.5                                  | +                              | w | - | - | - |
| Antithrombin III                   | 65 000           | ~0.3                                  | -                              | - | w | + | - |
| C1s inhibitor                      | 104 000          | 0.2-0.3                               | w                              | w | + | - | + |
| $\alpha_2$ -macroglobulin          | 820 000          | 2.0-3.5                               | +                              | + | + | + | - |

<sup>1</sup> Data from (2) and (4).

<sup>2</sup> + = strong, - = no, and w = weak inhibition.

a = trypsin, b = chymotrypsin, c = plasmin, d = thrombin, e = C1s inhibitor.

No comprehensive review on Achy has been found but some information exists in the following papers: Schultze & Heremans (1), Blombäck & Hanson (2), Putnam (3), Ritzmann & Daniels (4), Bianchi et al. (5), and Laurell (82).

Achy, initially described as  $\alpha_1$ X-glycoprotein by Schultze et al. (90), binds and inactivates chymotrypsin specifically. The fetal plasma level of Achy is low until birth, and the concentration increases rapidly during the first weeks of life. The newborn child has about 0.1 g/liter Achy and the adult normal plasma level is about 0.5 g/liter.

Achy is a strong acute phase reactant (APR), increasing rapidly in concentration in an inflammatory reaction. Together with CRP, Achy increases fastest of the APRs, giving values above the upper limit already 8 h after tissue lesions. The Achy levels during a disease have been followed for cholecystectomy (4,82), mastectomy (82), myocardial infarction (4,82) and for children with acute thermal burns (4).

Achy is a glucoprotein with a molecular weight of 69 000 (2). It is found in Cohn fraction IV<sub>4</sub> (1), together with other proteins, such as transferrin and haptoglobin.

### Statistical Methods

I used the "classical" procedure to estimate the regression line, assuming that the x-values were without error, when calculating the standard curves throughout this study.

When comparing results from two methods, I used the Deming's procedure (91,92) to estimate the regression lines. It was then unnecessary to use one of the methods as a reference not subjected to errors. Instead the square of the quotient between the random errors in the two methods

( $\lambda = \frac{S_e^2}{S_d^2}$ ), is used, where  $S_e$  and  $S_d$  are the random error values found

for method x and y respectively.

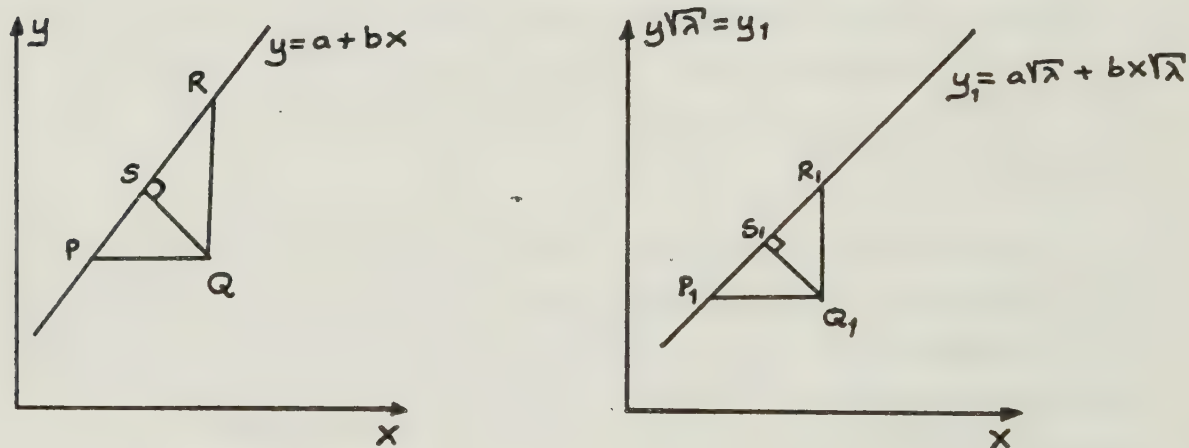


Figure 2. Simplified illustration of three ways to measure the distance from a measuring point (Q) to the estimated line  $y = a + bx$  when the method y is compared with the method x.

Both the classical and Deming's procedure utilise least squares analysis. The parameters of the line are calculated in such a way that the summation of all the squared distances from the measuring points to the

line attains a minimum. The classical procedure and Deming's procedure differ in the direction chosen to measure these distances.

The classical procedure with  $x$  as the independent variable assumes that no error exists in method  $x$ . The distances (squared) to be minimized are thus in the  $y$ -direction, i.e.  $\overline{QR}$  (Figure 2). If  $y$  is the independent variable, random error is supposed to be present in method  $x$  only. The distances (squared),  $\overline{PQ}$  (Figure 2), are then minimized.

For Deming's procedure the square of the distances  $\overline{QS}$  (Figure 2) shall be minimized. In order to find the direction of  $\overline{QS}$ , all  $y$ -values are multiplied by  $\sqrt{\lambda}$  thus making the random errors in both methods to be of the same magnitude, since  $\sqrt{\lambda} = S_e/S_d \Rightarrow S_e = S_d \cdot \sqrt{\lambda}$ . This means that the direction of  $\overline{Q_1S_1}$  will be perpendicular to the line. Then the line  $y = a + bx$  can be found, since  $a$  and  $b$  can be calculated from  $y_1 = \sqrt{\lambda}a + \sqrt{\lambda}bx$ .

Further details can be read in (92). Four recent papers (93-96) also discuss Deming's procedure, and all found it to be a most useful statistical tool.

By using the equations in Wakkers et al. (92), I set up a programme on a Hewlett-Packard 9820 A Calculator, which was used throughout this study.

## MATERIALS

### Samples

Serum samples used were those remaining after different routine tests within the laboratory. Many undefined disease states were thus represented. Some selection was also made, in order not to obtain too many "normal" samples, e.g. samples from the protein laboratory (electrophoresis, a few patients with low or no Hp, and other abnormal protein levels), and from the chemical laboratory (high and low TIBC, lipemic samples, hemolyzed samples, high levels of creatinine, urea, glucose etc.).

Serum samples used in the BCG-ESR study, were drawn in parallel with the sampling for ESR. Some patients, known earlier to have moderate or



high ESRs, were also selected in order to get an extension of the range in ESR, and to get a more uniform spread in ESR values.

Urine samples were first taken randomly from our "urine analysis laboratory". Later a selection was made in order to obtain a higher proportion of abnormal samples, e.g. high levels of glucose, ketones, blood (hemoglobin) as found by dip-sticks, and also high levels of urea and Bence-Jones proteins. Furthermore urines with strong colours were selected.

CSF samples used were randomly collected from the laboratory, after the routine tests had been performed, i.e. electrophoresis and total protein determination.

### Standards

Kabi protein standard (I) was used for albumin (BCG and immunological methods),  $\Delta$ C (BCG), and total protein determinations. This standard is a 100 g/liter solution of at least 98% pure human serum albumin, pH 7.3, containing no stabilizers or preservatives. This standard was diluted with distilled water to obtain a standard curve according to I-VII.

A serum pool was also used, consisting of a mixture of sera from 100 apparently healthy blood donors. Aliquots from this serum pool were transferred to small tubes and frozen. This pool was used as a standard (different dilutions) for the determination of all proteins, except albumin. This pool was expected to contain the mean normal levels of the different proteins. Furthermore a sample of this pool was analysed at an external laboratory (Department of Clinical Chemistry, University Hospital, Lund, Sweden) to check the levels of nine of the, for this study, most important proteins. The actual values used throughout my work, are given in the bottom line of Table 1 in paper I.

## METHODS

### Bromcresol Green Method, Immediate and Slow Reaction

The manual BCG method for determination of the content of albumin and slow reacting proteins in serum is described in (I). Two automated proce-

dures for serum albumin determination are presented in (IV).

The BCG reaction used for estimation of albumin in urine samples is presented in (II) with technical improvements in (VI).

Estimation of albumin by the BCG reaction with CSF (manual and automated procedures) is presented in (V).

#### Immunochemical Methods

Electroimmunoassay (EIA) was performed as described by Laurell (97). The runs were usually performed with a voltage of 10-12 V/cm gel for about 3 h. Some runs were however carried out overnight (3 V/cm for 16-24 h). Standards and samples (serum, urine or CSF) were diluted with electrophoresis buffer. The drying and staining procedures were carried out according to Axelsen et al. (98).

Crossed Immunelectrophoresis (99,100) was carried out as described by Weeke (pp. 47-56 in 98).

Single Radial Immunodiffusion (SRID) as originally described by Mancini et al. (33) and was performed according to a manual from the manufacturer of the antisera (Dakopatt A/S, Copenhagen, Denmark).

#### Electrophoretic Methods

Agarose Electrophoresis. The procedure described by Johansson (101) was followed. Further details in (I) and (VII).

Preparative Agarose Electrophoresis. The procedure described by Johansson (101) was followed. Further details in (I) and (VII).

Cellulose Acetate (CA) Electrophoresis. The manufacturer's prescribed technique for "Microzone" electrophoresis was mainly followed. Details in (I).

Polyacrylamide Gel Electrophoresis. This technique was used for the phenotyping of haptoglobin. A method was developed, based on LKB (Bromma, Sweden) Application Note No 306, with a specific staining procedure,

using first benzidine barium peroxide followed by treatment with naphthol yellow for permanent staining and better visibility. This method will be published separately. Further details in (VII).

### Chromatographic Methods

Gel Filtration was performed on Sephadex G 200 Superfine and Sepharose-CL 6B at room temperature. The columns used were i.d. 16 mm, length 1 000 mm with a bed height of 850 mm. Descending chromatography, in a 0.075 mol/liter barbital/Na-barbital buffer, pH 8.6, with 3 mmol/liter sodium azide, was performed at room temperature. 1 ml fractions were collected throughout this study. Each fraction was then individually analyzed for the different proteins indicated in (VII) and below.

Affinity Chromatography. The procedure used is described in (VII).

### Other Methods

Salting-out Experiments. A salting-out fractionation procedure using ammonium sulphate, according to Weeke (102), was performed. Details in (VII).

Haptoglobin Determination by a HbBC Method. Most Hp determinations were carried out with immunological methods. However in (VII) some results are estimated by the hemoglobin binding capacity (HbBC) method as described by Tarukoski (103).

Concentration and Desalting of Protein Fractions. Fractions from the preparative agarose electrophoresis, affinity chromatography and salting-out procedures were concentrated by using vacuum on collodium bags (VII).

## RESULTS AND DISCUSSION

### Albumin in Human Serum

As described earlier, this work was initiated due to the poor results obtained for albumin in our regional control programme. Correcting the standardization errors produced only a slight improvement. When the metho-



dological work began, all the laboratories used different modifications of the BCG method. A good documentation was found for the method developed by Doumas et al. (53), showing lack of possible interfering substances. However, by a more careful analytical technique and by using several pathological sera, I found (I), like others (56,58), that BCG gives artifactually high albumin values in the clinically important lower concentration range. Several factors were then investigated, such as dependence on pH, buffer concentration, Brij-35 concentration, temperature, BCG: sample ratio, etc.

It was found that the BCG reaction generally reached the "end point" much faster with albumin standard solutions than with serum samples. The time course of the reaction was followed for 60 min (I) using not only standards but also different types of serum samples (normal, newborn, and patients with different degrees of inflammatory response). The albumin concentration in the same sera was also determined by electroimmunoassay. A comparison showed that the zero-values (immediate reaction) were correct, while the values after 60 min were considerably overestimated.

In (I) it was clearly demonstrated that the immediate BCG method gave reliable albumin results which compared well with EIA and CA-electrophoresis, generally considered to be accurate and specific methods. This was also shown with grossly abnormal serum samples. In contrast, values obtained with the BCG method after 60 min reaction time differed considerably from these obtained with the other two methods.

Reaction times of 5 to 30 min had been used in all the work published previously (I). The resulting values then lie between the two extremes, 0 min and 60 min. Most of the slow reacting components react with BCG during the first 5 min. An example was presented in (I) indicating the considerable difference which could be obtained; 27 g/liter albumin at 0 min increased to 43 g/liter at 60 min (within the normal range!). EIA gave a value of 28.5 g/liter for this serum sample.

The results from the immediate BCG method were unaffected by moderate changes in pH (3.9-4.4) and temperature (20-45°C).

The specificity of the immediate reaction is further shown in (VII) by the good agreement with immunological methods in the chromatography experiments. This specificity has also been confirmed by several other authors (i.e. 59,61-64,71,72,75,81). Corcoran & Durnan (60) repeated this work and found the same results while Webster (59) found a fairly constant difference of 3.0 g/liter, but agreed on the existence of a fast and slow reaction. Webster postulated that other fast reacting nonalbumin material exists in serum. As discussed in (IV) and (VII) it seems more probable that this difference depends on the use of saline for the predilution of serum samples. The saline may cause one or more of the slow reacting components to react rapidly. The same should be true for (65). Ingwersen & Raabo (69) used a reagent with lower BCG concentration which reduces the binding of globulins to such a degree that the absorbance can be measured about 1 min after mixing serum with BCG reagent. However, they also prediluted the serum samples with saline, so that their method must be studied further.

The group around Savory have published three papers on serum albumin determination with BCG (63,64,70). In the first paper from 1976 (70) a predilution step with saline was performed. The reaction between BCG and albumin was stated to be less than 1 s (stopped-flow analysis), but no comments were made on the slow reaction. The reading time used was 30 s, giving (together with the predilution step) the typical overestimation in the low albumin range ( $y=5.90+0.84x$ ). The next paper from 1977 (63) referring to paper (I) demonstrated the improvement when using shorter reaction times (13 s). In the third paper (64) the immediate reaction was used in a flow-injection analysis system, referring only to (63).

As an example of the possibility to automate the immediate BCG method, paper IV describes its application on the LKB 2086 Reaction Rate Analyzer and the LKB 2480 Prisma multichannel analyzer with good results. Other automated equipment have also been shown to give good results with the immediate BCG method: Technicon SMA 12/60 or Auto/Analyzer II, Plesher (61), Technicon AutoAnalyzer II, Speicher et al. (72), Technicon SMAC, Cederblad et al. (62), centrifugal analyzer, King et al. (63) and flow-injection analysis system, Renoe et al. (64).

The influence of heparin on the BCG and BCP methods was earlier (53) shown to be negligible, but in two recent papers (104,105), using much higher heparin concentrations, some effects were noted. Bonvicini et al. (104) found a more pronounced effect on BCG (10 min reading time) than on BCP. Perry & Dumas (105) found a much smaller effect for BCG (too low values) than Bonvicini et al., whereas a more pronounced effect was found for BCP (too high values). The conclusion must be to preferably avoid or at least to be careful when using heparinized plasma in both methods. It was also concluded that heparin interference is not appreciable when administered in vivo, even at very high doses.

Some reports (46-48) state that the BCP method is more specific for albumin than the BCG method. These authors, however, compared their BCP method with a slow BCG method, giving falsely high albumin results in the low concentration range. Recently, Tel et al. (45), found that the BCP method was not as specific as has been claimed earlier.  $\alpha$ -,  $\beta$ -, and  $\gamma$ -globulin fractions react with BCP. Tel et al. prefer the BCG technique as its results are more comparable with those of other methods. Moreover, they found considerable deviations from the recommended values for non-human sera used in quality control and calibration procedures when using the BCP method. This is not the case with BCG (I). Pinnell & Northam (46) found that BCP underestimates the amount of serum albumin in icteric patients. This may possibly be due to the fact that bilirubin binds to albumin at the pH used for the BCP method. The non-interference by the BCG method (53) may be explained by the lower pH (4.2) used where bilirubin does not bind to albumin.

When selecting a routine method for serum albumin determinations, three groups of methods must be considered, i.e. electrophoretic, immunological and dye-binding techniques.

The electrophoretic methods have the disadvantages that they are not possible to automate and that the dyes used do not stain all proteins to the same extent (106). The latter creates problems for the estimation of albumin in pathological sera.

The immunological methods have the advantage of being very specific, if the antiserum is raised and checked appropriately, and they then show reasonably good precision. The SRID and EIA are difficult to automate. The



SRID is also affected by aggregates of albumin (81). The nephelometric methods are easily automated but the cost of antiserum is rather high.

The dye-binding methods are currently the most frequently used and they have the following advantages: low reagent and labour cost, rapid and simple to perform, easy to adopt for a large sample output in the ordinary clinical laboratory. The HABA and MO methods have been shown not to be specific for albumin. The BCP method has earlier been claimed to be fully specific, but certain interfering substances have recently been recognised (45,46). The BCP also gives a lower absorbance than the BCG method. It was earlier claimed that the BCG method was not entirely specific. With the introduction of the immediate BCG method (I) however, this problem has been overcome (I and VII). It is also possible to automate the method (IV and others, see above).

Thus the immediate BCG method, being as specific as the immunological methods (I,IV,VII), and with the advantages mentioned above and the better precision, should be the method of choice in most laboratories for the estimation of serum albumin concentration.

#### Albumin in Human Cerebrospinal Fluid

Axelsen et al. (98) demonstrated the determination of fifteen different plasma proteins in CSF. They concluded that the concentration of the individual proteins in the CSF is correlated to the concentration in serum and to the total protein content of the CSF. The CSF protein pattern can only be evaluated if the CSF total protein is known and the plasma protein pattern in blood is investigated by e.g. agarose gel electrophoresis. Blombäck & Hanson (2) voice the same opinion. They also claim that electrophoresis of CSF and/or quantitative determinations of single plasma protein components provide limited information beyond that obtained from the total protein determination. An important exception is electrophoretic or immunological determination of immunoglobulins in CSF and plasma, especially of IgG, because of local production caused by some diseases.

Due to the good correlation between albumin and total protein determinations (98), total protein could be substituted by albumin for detecting defects in the blood-brain barrier (albumin quotient). Albumin can be determined more accurately since the total protein methods all have some drawbacks e.g. different sensitivity to different proteins.

Albumin levels in CSF and serum are also needed to calculate the IgG/Albumin index, to identify any local IgG production in or near the subarachnoid space.

Due to the disadvantages of the total protein methods for CSF, it would seem more appropriate that the calculation of the protein content in the different fractions obtained by CA electrophoresis should be based on a separate albumin determination, rather than on the total protein content.

In order to simplify the determination of albumin in CSF an immediate BCG method was developed (V). The difficulties and disadvantages of electrophoretic and immunological methods have been discussed above. The immediate BCG method (y), as described in (V), shows a good comparison with electroimmunoassay (EIA) (x):

$$y = 16.9 + 0.995x \quad (n=51; r=0.995) \text{ mg/liter.}$$

An automated method (y), using LKB 2086 Reaction Rate Analyzer, was also developed and compared with the manual (x) technique:

$$y = 1.9 + 0.996x \quad (n=16; r=0.970) \text{ mg/liter.}$$

After albumin, IgG has the next highest concentration among the proteins in CSF, and future work may reveal if it is possible to use a new globulin/albumin index, calculated as the quotient of the CSF/serum ratios of globulins (as the difference: total protein minus albumin) and albumin.

The immediate BCG method could then be combined with the simple CSF total protein method using Ponceau S, developed by Pesce & Strande (107) and modified by Salo & Honkavaara (108).

### Albumin in Human Urine

An increasing interest in the determination of specific proteins in urine has been noted lately since all total protein methods available now have certain drawbacks, such as different sensitivity to the different proteins, and also precision problems. Thus, it should be possible to replace total urinary protein by the estimation of urinary albumin.

The immediate BCG method was modified and used for urinary albumin determination in (II). Modifications were made in (VI) in order to simplify

the procedure. An initial adjustment of the pH of the urines was necessary. First a pH indicator strip was used, but later two indicators, phenol red and bromthymol blue which did not bind to albumin and thus did not disturb the albumin-BCG reaction were found. These indicators were also selected so that they did not contribute to the absorbance at 629 nm at pH 4.2. They were mixed with the urine samples prior to pH adjustment.

The comparison of the initial immediate BCG method (y) with EIA (x) was (II):

$$y = 17.2 + 1.006x \quad (n=98; r=0.99) \text{ mg/liter.}$$

The methods using the two indicators (phenol red and bromthymol blue) compared well (VI) with the first method.

These methods (II and VI) were designed to ascertain the albumin concentration in patients with proteinuria. The intention was to use it together with serum albumin (I) to obtain the albumin clearance and to follow changes in urinary protein during pregnancy. The purpose was not to discover just slightly increased or normal levels of albumin in urine when a different sample/reagent ratio should be chosen.

The overall performance when using the 0-min BCG method was only slightly better than the 60-min BCG method. Nevertheless, in some urines, large differences were found, ranging from a 50 to 100% false increase in the 60-min values.

In a future study, it will be investigated if this difference ( $C_{60\text{min}} - C_{0\text{min}}$ ) can be used to ascertain the degree of selectivity for a glomerular proteinuria. The proteins causing the slow BCG reaction are transferrin, haptoglobin and  $\alpha_1$ -antichymotrypsin.

The choice of which method, the immediate BCG method or nephelometric methods with (109) or without (110,111) polymer enhancement, should be used in the individual laboratory depends of which method fits best into the routines. Both techniques give reliable results and are easy to automate. The reagents for BCG cost less.



### The Influence of Citrate and Phosphate on the SRID Technique

When working with the immediate BCG method for urinary albumin determinations (II), a correlation study with the SRID technique showed large disparities between the two methods (III). SRID gave falsely high albumin results not only for untreated urines but also when known amounts of albumin were added to urine samples as well as to a "synthetic urine". On investigating the different components of the "synthetic urine", it was found that the disparities were due to the influence of citrate and phosphate (III). On the addition of citric acid or phosphate to the dilution buffer and/or the gel buffer, the results of the SRID method agreed with those of other methods, BCG and EIA, and with the expected results.

The enlarged precipitate rings and colour effects (III) indicate that these problems arise either indirectly by a binding to the immunoglobulins in the antiserum with a following change in the antibody binding properties, or directly by affecting the antigen-antibody reaction. The weak bindings between the immunoglobulins and the agarose gel may also be affected. The fact that calcium has a strong affinity to both citrate and phosphate, may also have an effect.

This may be a general phenomena in immunological methods, or it may be limited to this specific situation or to a limited number of other analytical situations.

### The Slow BCG Reaction and Its Use (Acute Phase Reactants)

The measure of the slow BCG reaction is defined as the difference between the "albumin" concentration obtained after 60 min and 0 min reaction time. This is called  $\Delta C$  and is expressed in g/liter (VII). In paper (I),  $\Delta A\%$  was used instead. This is defined as the percentage of  $\Delta A$  for the actual sample compared with  $\Delta A$  for a large serum pool (defined as 100%).  $\Delta A$  is the absorbance difference between 60 min and 0 min.

$$\text{Thus: } \Delta A\% = \frac{\Delta A_{\text{sample}}}{\Delta A_{\text{pool}}} \times 100$$

In the first paper (I) it was demonstrated that  $\Delta A\%$  may be used as a measure of acute phase reactants in serum.  $\Delta A\%$  showed a good correlation with

some acute phase proteins and with the  $\alpha_2$ -globulin fraction from CA electrophoresis. A table and figures in (I) show these results. In (I) a suggestion was also made how this information could be used. In a diagram the two measures of the BCG method were combined,  $\Delta A\%$  on the y-axis and albumin on the x-axis. Although the lines in the figure are rather arbitrary and not intended to be definite borderlines, it seemed to be possible to classify patients according to the degree of their inflammatory reaction. Further results and discussion can be seen in the next paragraph.

Several techniques were used (VII), including analytical and preparative agarose electrophoresis, gel and affinity chromatography, comparison of different patient's sera, Cohn fractions, salting-out procedures, and the use of commercial purified protein preparations, in order to identify the slow reacting components. Three serum proteins, Tf, Hp and Achy reacted slowly with BCG. No other of the major serum proteins reacted slowly with BCG. Only albumin reacted immediately.

$\Delta C$  (or  $\Delta A\%$ ) can be used as a measure of "acute phase reactants" as described in (I), since the increase in the Hp and Achy concentrations in sera of patients with an inflammatory reaction is much more pronounced than the resulting decrease of the Tf concentration (82). Hp and Achy can reach values up to eight times the normal concentration, while Tf decreases to about 70% of normal. This means that  $\Delta C$ , calculated with the factors given in (VII) reaches about 16-17 g/liter (corresponding to  $\Delta A\%$  of about 310-330% of normal concentration), while normal sera have a mean of 5.1 g/l. These levels were also reached and exceeded in some pathological sera during my work with this method.

It was also shown (VII) that Tf and Achy react with BCG at the same molar ratio as albumin. For Hp twice as much BCG will react, indicating that each of the two subunits (either the two  $\alpha$ - or the two  $\beta$ -chains), react with BCG as albumin does.

It is thus possible with the very simple BCG method to simultaneously obtain both a reliable albumin value and a measurement of "acute phase reactants" in serum. This may be of importance in several diagnostic and prognostic situations.



### ESR Compared with Immediate and Slow BCG Reaction

Fåhræus (112) showed that the rate of sedimentation for erythrocytes (ESR) is related to the degree of activity for different disease processes. Schultze & Heremans (1) summarize the different factors contributing to the ESR. The main factor responsible for the acceleration of the ESR is fibrinogen, but also other serum components contribute e.g. gamma globulins, agglutinins and  $\alpha_2$ -macroglobulin. Hp, IgG and ceruloplasmin are moderately active too. Several other serum constituents also contribute, e.g. bile constituents, pharmaceutical substances, heparin, dextran etc.

Since both  $\Delta C$  (above) and fibrinogen are APRs, they generally show the same pattern of changes in different disease states. Since fibrinogen is the main factor for the acceleration of the ESR,  $\Delta C$  can be expected to parallel the ESR in at least uncomplicated acute inflammatory diseases. Immunoglobulins are the second factor which alter the ESR. Immunoglobulins generally rise in diseases lasting for at least about two weeks, while albumin shows a slow decrease. This should mean that in chronic disease states the increase in ESR should be inversely proportional to the albumin level.

These hypotheses were tested by estimating ESR, albumin,  $\Delta C$  and total protein content in 37 patients, selected as described under MATERIALS.

In Figure 3, the  $\Delta C$  (slow BCG reaction) was compared with ESR. The normal range for  $\Delta C$ , 5.1 g/liter  $\pm 20\%$  (4.1-6.1 g/liter), has been used earlier (I) but was then expressed as  $\Delta A\%$  (80-120%). The normal ranges used for ESR are:

|        | < 50 years old<br>(mm/h) | > 50 years old<br>(mm/h) |
|--------|--------------------------|--------------------------|
| Male   | 2-15                     | 2-24                     |
| Female | 2-22                     | 2-32                     |

It can be seen that  $\Delta C$  increased when ESR increased, but there were some patients, who showed increased ESR with normal  $\Delta C$ -values. This may be because other factors than fibrinogen affect the ESR (e.g. chronic diseases).



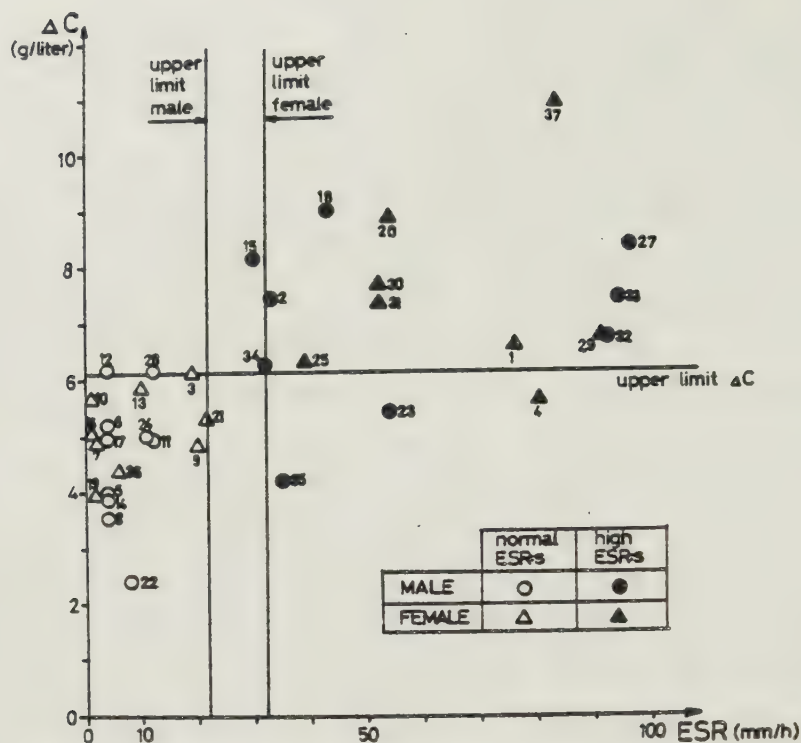


Figure 3.  $\Delta C$  compared with ESR for some patients' samples, some normal and some with unspecified, unselected disorders. (No:s at the points indicate patient numbers).

In order to use all the information obtained by the BCG method (immediate and slow reaction), the  $\Delta C$  and albumin values are shown in Figure 4.

An improved although still incomplete separation of normal and pathological levels was achieved. Probably patients with acute inflammatory diseases are those with high  $\Delta C$ -values, but only slightly decreased albumin values. Patients with low albumin but normal or only slightly increased  $\Delta C$  values are probably those with chronic diseases. However, one patient (No 4) with an ESR of 80 mm/h has normal values for  $\Delta C$  and albumin. This patient could have an M-component which gives an increased ESR, but does not affect the BCG method (immediate or slow), or a complication with intravascular hemolysis causing very low Hp levels despite an inflammatory reaction.

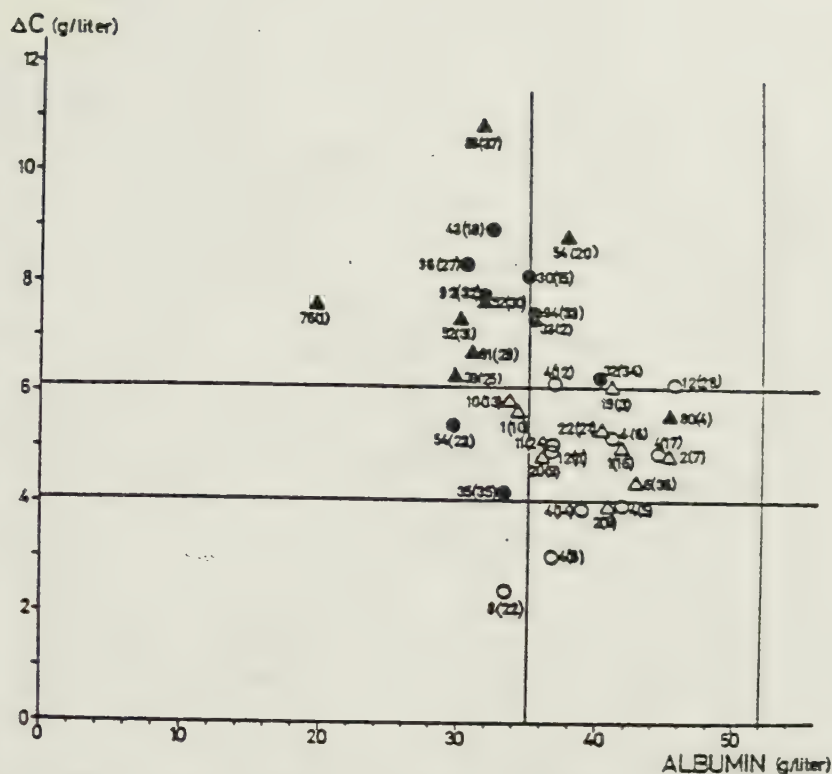


Figure 4. Relation between values for albumin and  $\Delta C$ , obtained by the BCG method, for the same patients as in Figure 3. (Numbers close to the points: e.g. 76 (1) indicates an ESR value of 76 mm for patient No 1).

Besides albumin, immunoglobulins are the major protein components in serum. Since the ESR increases with high immunoglobulin levels (chronic diseases or M-components), an attempt was made to correct for this. The difference between the total protein (TP) and albumin content was calculated. This difference was plotted on the x-axis and  $\Delta C$  on the y-axis in Figure 5.

An almost complete separation between normal and abnormal ESR values for patients in this study was now obtained. The diagram can be divided into four parts, ① - ④. Patients within the normal ranges for ESR,  $\Delta C$  and TP-albumin lie in section ①. Section ② probably contains patients with an acute inflammatory reaction, while section ④ probably contains patients either with a chronic disease or with an M-component. Section ③ is a mixture of the two types above. Patient No 4 with a high ESR, as discussed earlier, probably has an increased immunoglobulin (M-component?) level and not intravascular hemolysis resulting in low Hp-levels.

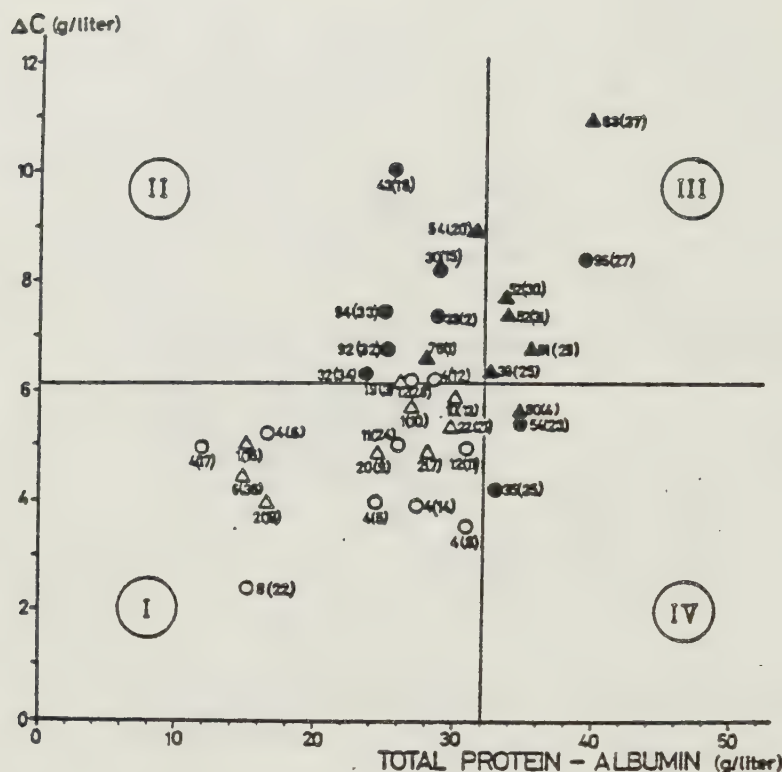


Figure 5.  $\Delta C$  compared with the difference between the total protein and albumin content. The same patients are plotted as in Figure 3. (Numbers at the points are as in Figure 4).

It is generally considered that single measurements of ESR, APRs or other proteins on a patient are of limited value, but serial determinations during a disease can provide much more information. Figure 6 gives hypothetical examples of the course of a few types of diseases.

The first hypothetical example (1) may be a patient with an acute inflammatory reaction with no further complications (i.e. surgical trauma, myocardial infarction etc. with complete recovery). The second curve (2) illustrates a patient with some later complications causing a rise in immunoglobulin and a decrease in albumin levels with later recovery. The third type of curve (3) may appear for patients with e.g. M-components.

However, recalculation of the results from Johansson et al. (113), using the factors in (VII) gives the following pattern for patients with and without complications in myocardial infarction after 1, 5 and 21 days. " $\Delta C$ " is then calculated from Tf, Hp and Achy values. Since the total protein values are not available, the total content of immunoglobulins (IgG + IgM + IgA) is used on the x-axis.



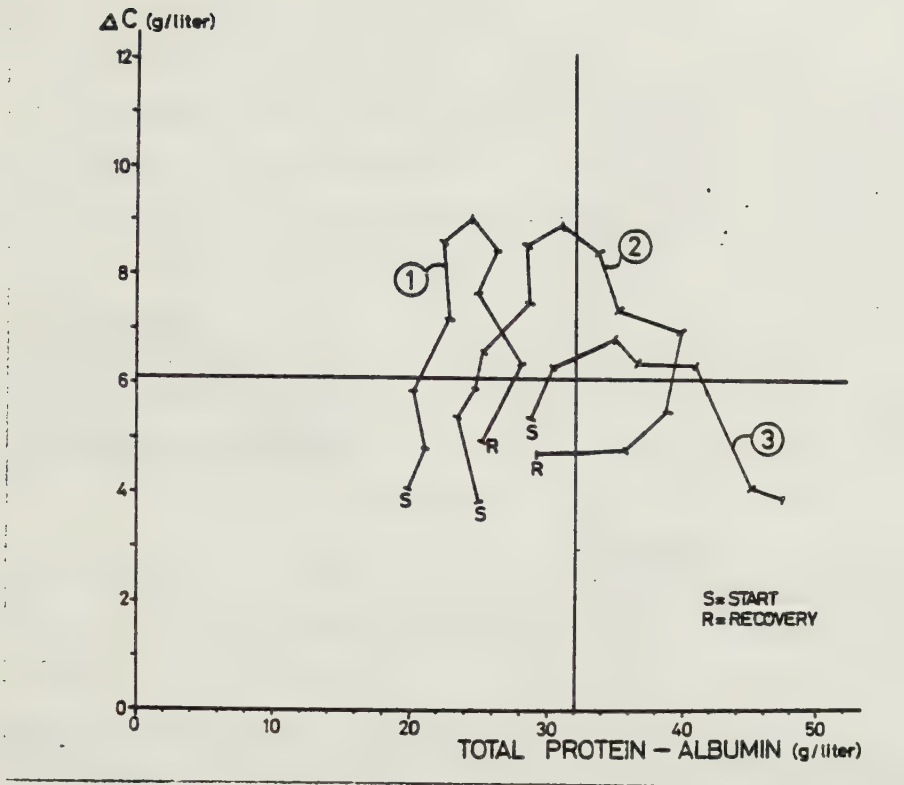


Figure 6. Hypothetical examples of the course of three types of diseases (S=start; R=recovery).

These results (Figure 7) indicate that the assumptions made above may be correct. If so, more information can be obtained by combining BCG (albumin and slow reacting components) and total protein values than by ESR alone. Thus two simple methods may substitute or at least add important information to ESR determination. Furthermore, it is indicated below that it is possible to make Hp, Tf and Achy to react quickly with BCG. This gives a considerable time saving, also in comparison with ESR determinations.

Of course the assumptions above are only hypothetical, but future studies, on different patient groups, may well lend support to them.

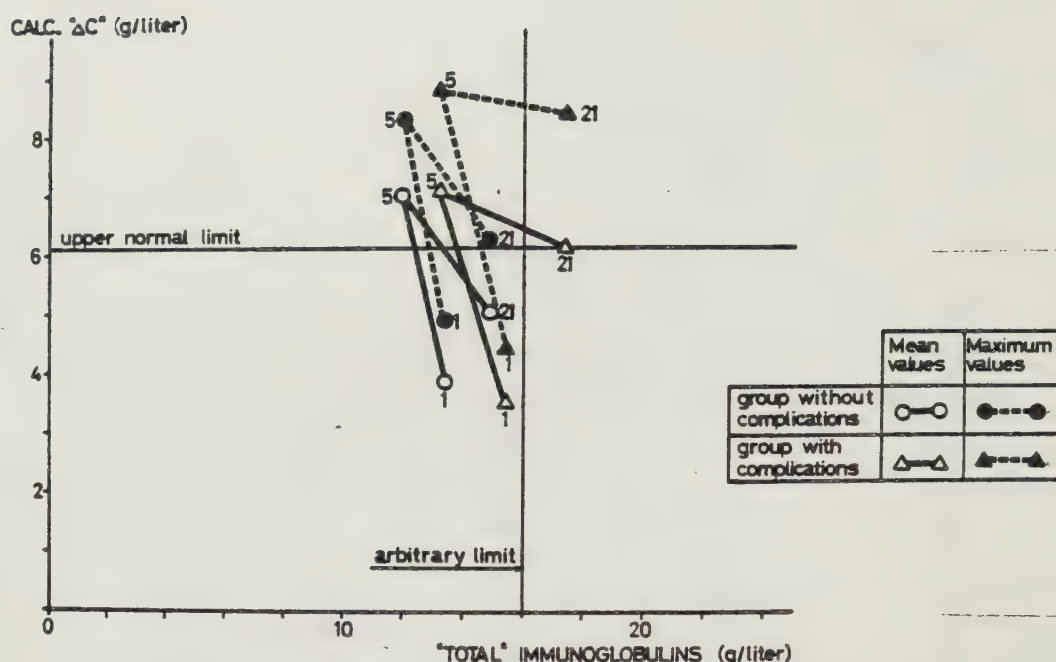


Figure 7. Recalculation of earlier published results (113) for patients with myocardial infarction (Calc. " $\Delta C$ " is calculated from Tf, Hp and Achy values using Table 6 in VII; since TP values are not available, "Total" immunoglobulins (IgA+IgG+IgM) was calculated). Numbers at the points are the day of admission (1) and then on days 5 and 21.

### Other

No efforts have been made here to show the nature of the slow BCG reaction. Hp, Tf and Achy may have to undergo slow conformation changes (rearrangements of the molecules) before they can react with BCG. The following facts may point in this direction. It was discussed in paper II that if the samples were adjusted to about pH 4 before being mixed with BCG reagent, these three proteins then reacted immediately. The same was true if the samples were prediluted in physiological saline solution before mixing with BCG reagent (IV). It was shown by Putnam (3) that Hp was denaturated to about 80% within about 30 min at pH 4.2. The complex between Tf and Fe (III) is not stable on acidification to pH 4 (3), resulting in apotransferrin which is much less resistant to denaturation than the Tf-Fe (III) complex. No such data are available for Achy.

## GENERAL SUMMARY

The work on this thesis was started due to the large variations in serum albumin results between the laboratories participating in the regional control programme. After minimizing the standardization error it was shown that the main error remained. I then started the methodological work, which resulted in the immediate BCG method but also in the other findings presented in this thesis.

The reaction between BCG and different body fluids, e.g. serum, urine and CSF, has been studied. It was found early on that the reaction between BCG and serum proceeds in two steps; albumin causes the immediate reaction while other proteins cause the slow reaction.

The immediate BCG reaction with serum samples was found to be specific for albumin. This was shown by comparing BCG results for different sera with results from immunological methods such as SRID and EIA. Moreover the values determined by both BCG and immunological methods on the fractions from gel chromatography (Sephadex G 200 and Sepharose-CL 6B) were almost identical. The earlier claimed unspecificity of BCG for albumin, resulting in spuriously high results in the important low albumin concentration range, was shown to depend on too long reaction times. The slow reacting components (APRs) were then estimated together with albumin.

It is concluded that the immediate BCG method at present ought to be the method of choice in most laboratories for the estimation of serum albumin concentration.

The immediate BCG method was also, with some minor modifications, adopted for albumin determinations in urine and CSF. Good comparisons with immunological methods were obtained.

When studying the immediate BCG method for urinary albumin determination, a comparison with SRID was performed. The latter gave erroneously high results not only for urines but also when known amounts of albumin were added to urine samples as well as to a "synthetic urine". It was found that the disparities were due to the influence of citrate and



phosphate. A means of overcoming this problem is given.

Using several techniques the components reacting slowly with BCG were shown to be Hp, Tf and Achy. The measure of the slow BCG reaction is defined as the difference between the "albumin" concentration obtained after 60 min and 0 min reaction time. This is called  $\Delta C$  and is expressed in g/liter. However, in the first paper  $\Delta A\%$  was used. This is defined as the percentage of  $\Delta A$  for the actual sample compared with  $\Delta A$  for a large serum pool (defined as 100%).  $\Delta A$  is the absorbance difference between 60 min and 0 min. It was shown that  $\Delta C$  (or  $\Delta A\%$ ) was a measure of the acute phase reactants in serum. The two values obtained by the BCG method, albumin and  $\Delta C$ , can be useful in several diagnostic and prognostic situations.

ESR was compared with results from the immediate and slow BCG reactions. The most useful information was obtained by combining the BCG method with a total protein determination. In a diagram  $\Delta C$  (y-axis) was compared with the difference (x-axis) between total protein and albumin values. For the patients in this study a complete separation between normal ESR:s and pathological ESR:s was obtained. In addition other information, not obtainable by ESR, can be of clinical value. This must however be checked by future studies on patients with different diseases followed during the course of their disease. Some earlier published results for patients with myocardial infarction were recalculated. These results show that this approach may be fruitful.

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## Improved Specificity of Serum Albumin Determination and Estimation of "Acute Phase Reactants" by Use of the Bromcresol Green Reaction

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The reaction of serum samples with bromcresol green proceeds in two steps. Albumin is responsible for the faster (<1 min) reaction; the slower (30-min) reaction is a measure of "acute phase reactant(s)" in serum. Serum is simply mixed with bromcresol green reagent and the absorbance is measured twice, immediately and at 60 min. Albumin concentrations, determined from the absorbance at 0 min, correlate well with those determined by Laurell "rocket" immunoelectrophoresis;  $r = 0.95$  with no certain deviation from unity for the slope and with a negligible difference at zero concentration. The slow reaction was expressed as  $\Delta A\% = 100 (\Delta A_s / \Delta A_v)$  where  $\Delta A_s$  and  $\Delta A_v$  are the changes in absorbance between 60 and 0 min for the sample and a commercial control serum, respectively. The value for  $\Delta A\%$  correlates well with the percentage of  $\alpha_2$ -fraction as determined by electrophoresis on cellulose acetate, as well as with orosomucoid and ceruloplasmin, all of which are acute phase reactant(s). Whether these proteins or other acute phase reactant(s) actually cause the slow reaction has not yet been established.

Many methods have been described for determining albumin in serum (1-17), among them various dye-binding methods such as 2-(4'-hydroxyazobenzene)-benzoic acid (HABA) (6-8), methyl orange (9, 10) and bromcresol green (BCG) (11-17).

The dye-binding methods are simple, rapid, suitable for automation, and can be routinely used for many samples in the ordinary clinical laboratory. The reagents are easy to prepare, inexpensive, and stable. However, it has been claimed that they are not very specific. The bromcresol green method has been investigated by several authors, and although its specificity is better than that of another commonly used method (13), for example, it has only been recommended as a screening method (14-16). Results by the bromcresol green

method do not compare well with more specific methods for albumin determination, especially in the clinically important lower concentration range (15, 17), the BCG method giving artifactually high values for such sera. In contrast, agreement is good for albumin concentrations near 40 g/liter.

Our own experience with different BCG methods suggests that the method described by Doumas et al. (12) is superior. The standard curve is linear, with suitable absorbances of the final solutions over the desired concentration range. Moreover, these authors evaluated the susceptibility of the method to possible interfering substances.

We made the fundamental observations reported here during an attempt to standardize the BCG method within a laboratory group in Sweden. The aim of our studies was to optimize the standardization and to improve the performance of the method.<sup>1</sup> We demonstrate here that the reaction of BCG with serum samples proceeds in two stages, one fast and one slow; albumin causes the fast reaction and the slow reaction is with other serum proteins.

### Materials and Methods

#### BCG method

**Reagents.** The reagents were those for the manual method described by Doumas et al. (12). Bromcresol green from Hopkin & Williams Ltd (Romford RM1 1HA Essex, England) was used.

Kabi protein standard (KABI AB, S-104 25 Stockholm, Sweden) was used. This standard is a 100 g/liter solution of at least 98% pure human serum albumin, pH 7.3, containing no stabilizers or preservatives. This standard was diluted with distilled water to contain 20, 40, and 60 g/liter.

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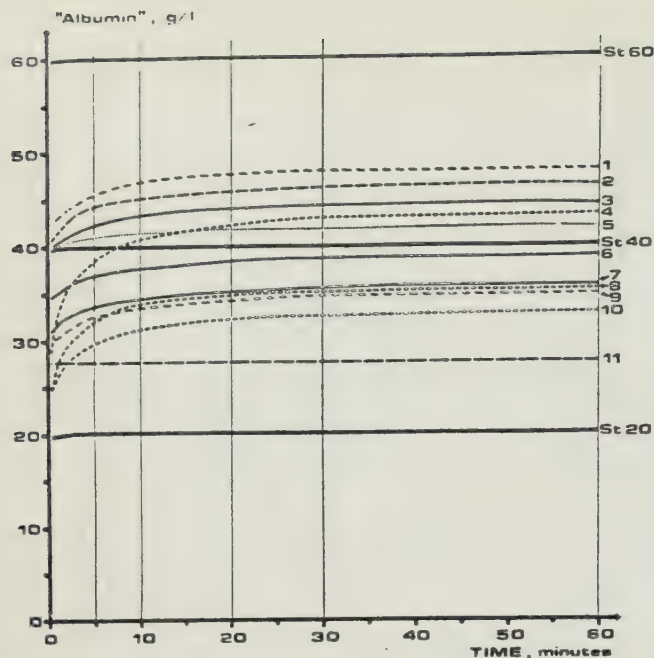


Fig. 1. The time course of color development with BCG for different samples

Apparent "albumin" values (g/liter) on the ordinate. St20, St40, and St60 are pure human albumin standards, 20, 40, and 60 g/liter, respectively. 11, albumin fraction from preparative electrophoresis; 3, 6, 7, normal patients' sera; 2, patient serum with a slight inflammatory response; 10, patient serum with a moderate degree of inflammatory response; 4, 8, patient sera with intense inflammatory response; 5, newborn; 1, human control serum (Versatol); and 9, horse control serum (Seronorm)

The commercial lyophilized human control serum we used was "Versatol" (General Diagnostics, Morris Plains, N. J. 07950) and the commercial lyophilized equine control serum used was "Seronorm" (Nyegaard & Co AS, Oslo, Norway).

**Apparatus.** Absorbances were measured at 629 nm with a Model 124 spectrophotometer (Coleman Instruments Division, Maywood, Ill. 60153) equipped with an original water-thermostatted sample cell assembly set at 25 °C, and a recorder with a balancing time of less than 0.5 s/250 mm.

**Procedure.** Pipet 2.0 ml of dye solution<sup>2</sup> into a measuring cuvette and into a blank cuvette. Add 10  $\mu$ l of the serum sample or standard to the measuring cuvette with a disposable capillary micro pipette and stir immediately. Note the exact time of mixing. Follow the increase in absorbance for 2–3 min on the recorder before the next sample is measured. After 60 min at room temperature, again measure the absorbances of all standards and sera.

### Electroimmunoassay (EIA)

We used the Laurell (4, 18) "rocket" technique. We added antihuman albumin antiserum to a 10 g/liter solution of buffered Indubiose A37 Agarose (L'Industrie Biologique Francaise S.A., Gennevilliers, Seine, France) at 53–54 °C.

<sup>2</sup> Per liter, 0.15 mmol of BCG, 75 mmol of sodium succinate, and 4.0 ml. of "Brij 35" surfactant (300 g/liter); pH 4.20  $\pm$  0.05.

The rabbit antihuman albumin serum was obtained from Dr. B. G. Johansson, Department of Clinical Chemistry, University Hospital, S-221 85 Lund, Sweden. The specificity of the antiserum was tested by crossed immunoelectrophoresis with intermediate gel (18, 19). The intermediate gel contained the antiserum to be tested and the rest of the plate was covered with antihuman (polyvalent) serum (Dakopatt A/S, DK 2900 Hellerup, Denmark). When a human serum sample was run, only trace amounts of impurities were found in the anti-albumin serum, and its specificity against human albumin had been clearly shown.

Standards (Kabi, 20 to 60 g/liter) and sera were diluted 200-fold with the electrophoresis buffer, a barbital/sodium barbital buffer (75 mmol/liter, pH 8.6, containing 2 mmol of calcium lactate per liter) as described elsewhere (18, 19).

Of the diluted samples, 5  $\mu$ l was applied to each of the 2.5-mm-diameter wells with a Hamilton syringe, and electrophoresed at 10 V/cm for 3 h. The subsequent drying and staining procedures were carried out according to Axelsen et al. (19). Peak heights were measured and plotted and the resulting standard curve was used to ascertain albumin concentration in the samples.

The other proteins were similarly determined, rabbit antihuman serum against orosomucoid (from Dr. B. G. Johansson), ceruloplasmin, haptoglobin, and hemopexin (Dakopatt A/S) being used.

### Electrophoresis on Cellulose Acetate (CA)

We followed the manufacturer's prescribed technique for "Microzone" electrophoresis (Beckman Instruments Inc., Fullerton, Calif. 92634), with the following exceptions. "Sepraphore III" membranes were used (Gelman Instrument Co., Ann Arbor, Mich. 48106) and the fixation, staining, clearing, and drying procedures were changed accordingly. The protein fractions were quantitated with an automatic scanner (Camag, CH-4132 Homburger Strasse 24, Switzerland) equipped with an electronic integrator, printer, and recorder.

Total protein concentration in the samples was determined by a biuret method on an AutoAnalyzer I system (Technicon Instrument Corp., Tarrytown, N. Y. 10591; method no N-14b) with Seronorm (Nyegaard & Co A/S) as the calibration material.

### Preparative Agarose Electrophoresis

This was done as described by Johansson (20). About 0.8 ml serum or plasma could be processed in each run. The purity of the albumin fraction was controlled by means of analytical separation by electrophoresis on agarose according to Johansson (20).

### Results

#### Some Analytical Variables

**Rate of the serum/bromocresol green reaction.** Figure 1 illustrates the time course of the increase in absorbance seen with various standards and serum samples. All curves were followed on the recorder from about 5



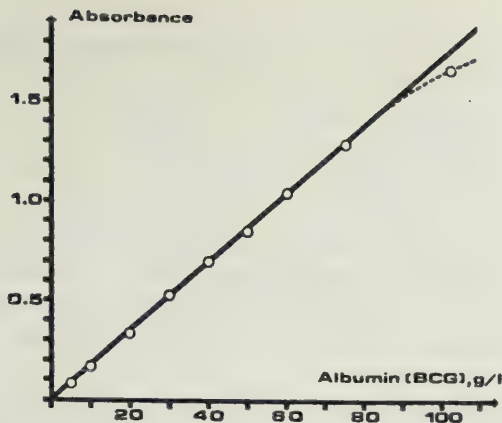


Fig. 2. Standard curve of albumin determination by the BCG reaction at 0 min

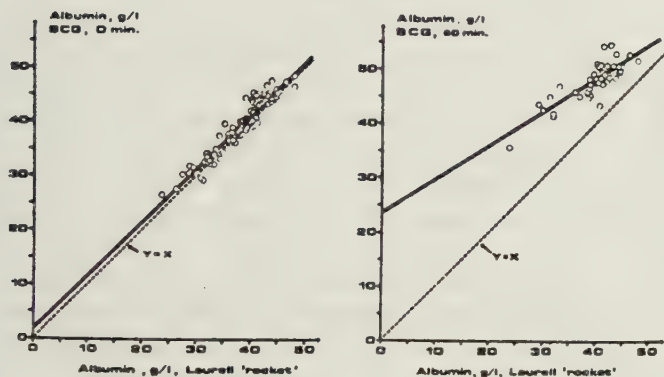


Fig. 3. Results by the BCG method at 0 (left) and 60 min (right) reaction times are compared to results by "rocket" immunoelectrophoresis

(left)  $y = 1.83 + 0.98x$  ( $n = 90$ ;  $r = 0.95$ )  
(right)  $y = 23.56 + 0.62x$  ( $n = 42$ ;  $r = 0.84$ )

s after mixing. The values on the y-axis are the values obtained with albumin as standard. Thus the values that would have been obtained at a given time by conventional methods of standardization can be seen.

Pure albumin standards and the albumin fraction obtained from preparative electrophoresis (Figure 1, no. 11) reach their final value in less than 1 min. In contrast, sera both of human and animal origin showed not only this fast reaction but also a much slower one, which reaches a steady value within 30 min at room temperature, although there is a considerable variation in the contribution to the total absorbance from the slow-reacting component(s) with different sera. Sera from patients with acute illness tended to show a more pronounced slow-reacting phase.

**Estimation of fast- and slow-reacting components.** The contribution to the absorbances from the fast reaction were estimated by extrapolating the recorder curve to the time of mixing, 0 min. The absorbance difference between 0 and 60 min was calculated and called  $\Delta A$ .

An arbitrary unit of the slow-reacting component is calculated from  $\Delta A\% = 100(\Delta A_{\text{sample}}/\Delta A_v)$  where  $\Delta A_v$  is the  $\Delta A$  for Versatol. Versatol was chosen for conve-

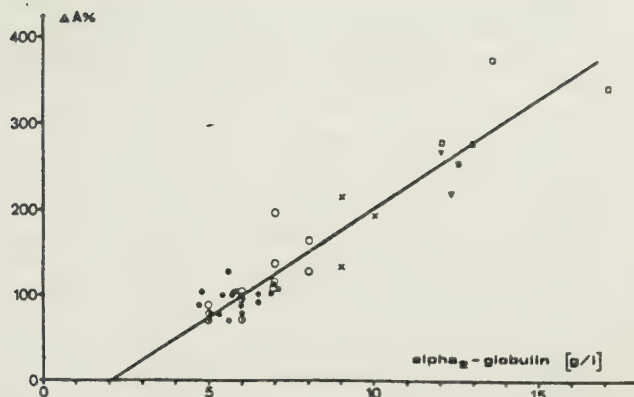


Fig. 4. Comparison of the slow reaction of the BCG method, expressed as  $\Delta A\%$ , and the  $\alpha_2$ -globulin fraction from cellulose acetate electrophoresis

The regression line was:  $y = -51.7 + 25.7x$  ( $n = 39$ ;  $r = 0.94$ ). ● = normals (blood donors); ○ = normals from routine electrophoresis; × = patients with a slight inflammatory reaction; ▽ = patients with a moderate inflammatory reaction; □ = patients with intense inflammatory reaction; ○ = newborns  
\* indicates chronic inflammatory reaction of corresponding degree

nience as the value for the batch we used agreed well with that found for a serum pool from healthy blood donors.

**Linearity and sensitivity of the BCG method.** A standard curve with albumin read at 0 reaction time (Figure 2) is linear up to 75 g of albumin per liter, with a sensitivity suitable for most photometers.

#### Comparison with Other Methods

Figure 3 shows results obtained with different patients' sera by the BCG method at 0 and 60 min, respectively, as compared with corresponding results by "rocket" immunoelectrophoresis. The linear regression curves and correlation coefficients ( $r$ ) calculated by classical technique assuming no variability in  $x$  or reference measurement were:  $y$  (BCG, 0 min) =  $1.83 + 0.98x$  (in g/liter;  $n = 90$ ;  $r = 0.95$ ) and  $y$  (BCG, 60 min) =  $23.56 + 0.62x$  (in g/liter;  $n = 42$ ;  $r = 0.84$ ).

The slope and intercept were recalculated by Deming's procedure as described by Wackers et al. (21). Assuming  $\lambda = 1$  (equal standard deviations of the methods) the regression curve is modified to:  $y$  (BCG, 0 min) =  $-0.04 + 1.03x$  (in g/liter;  $n = 90$ ). Results were similar when the BCG method at 0 and 60 min was compared with cellulose acetate electrophoresis. The following equations describe this comparison:  $y$  (BCG, 0 min) =  $2.31 + 1.01x$  (in g/liter;  $n = 110$ ;  $r = 0.91$ );  $y$  (BCG, 60 min) =  $21.71 + 0.60x$  (in g/liter;  $n = 57$ ;  $r = 0.84$ ).

Recalculation by Deming's procedure (as above) will give:  $y$  (BCG, 0 min) =  $-2.01 + 1.13x$  (in g/liter;  $n = 110$ ).

Results by the BCG method compare well with those by more specific methods for albumin determination when zero-minute values are estimated. Considerably higher values are obtained at 60 min, especially for patients' sera of subnormal albumin concentration. With a reaction time between these two extremes, intermediate results are obtained.

Table 1. Comparison of  $\Delta A\%$  with Concentrations of Some Proteins in Human Serum Samples<sup>a</sup>

| Code <sup>b</sup> | $\Delta A\%$<br>(%) | Oroso-<br>mucoid<br>(%) | Cerulo-<br>plasmin<br>(%) | C3<br>$\beta_1A-\beta_1C$<br>(%) | Hapto-<br>globin<br>(%) | Hemo-<br>pexin<br>(%) |
|-------------------|---------------------|-------------------------|---------------------------|----------------------------------|-------------------------|-----------------------|
| N                 | 71                  | 64                      |                           | 103                              | 44                      |                       |
| Nb                | 72                  |                         |                           |                                  | 15                      |                       |
| Nb                | 72                  | 69                      |                           |                                  | 24                      |                       |
| Nb                | 73                  | 36                      | 80                        | 155                              | 23                      | 54                    |
| N                 | 78                  | 66                      |                           |                                  | 67                      |                       |
| N                 | 78                  | 84                      |                           |                                  | 39                      |                       |
| N                 | 88                  | 89                      |                           |                                  | 79                      |                       |
| N                 | 88                  | 109                     |                           |                                  | 97                      |                       |
| $\alpha N$        | 89                  | 117                     |                           |                                  | 17                      |                       |
| N                 | 92                  | 114                     |                           |                                  | 79                      |                       |
|                   |                     |                         |                           |                                  |                         |                       |
| Nb                | 98                  | 73                      | 93                        | 217                              | 21                      | 68                    |
| N                 | 100                 | 97                      |                           | 105                              | 73                      |                       |
| N                 | 100                 | 110                     |                           |                                  | 99                      |                       |
| N                 | 101                 | 116                     |                           |                                  | 129                     |                       |
| N                 | 102                 | 100                     | 113                       | 136                              | 79                      | 110                   |
| N                 | 102                 | 100                     |                           |                                  | 109                     |                       |
| $\alpha LC$       | 102                 |                         | 93                        | 93                               | 18                      | 92                    |
| N                 | 103                 | 77                      | 93                        | 116                              | 147                     | 82                    |
| N                 | 104                 | 86                      |                           |                                  | 80                      |                       |
| N                 | 105                 | 93                      |                           | 127                              | 121                     |                       |
|                   |                     |                         |                           |                                  |                         |                       |
| N                 | 105                 | 107                     |                           |                                  | 144                     |                       |
| N                 | 109                 | 141                     |                           | 134                              | 126                     |                       |
| N                 | 114                 | 109                     |                           |                                  | 147                     |                       |
| N                 | 114                 |                         |                           |                                  | 156                     |                       |
| N                 | 117                 |                         |                           |                                  | 165                     |                       |
| N                 | 118                 |                         |                           |                                  | 162                     |                       |
| N                 | 128                 | 114                     |                           |                                  | 120                     |                       |
| LC                | 134                 |                         | 139                       | 161                              |                         | 101                   |
| Control           | 137                 | 143                     | 131                       | 126                              |                         | 100                   |
| P                 | 138                 |                         |                           | 165                              |                         |                       |
|                   |                     |                         |                           |                                  |                         |                       |
| L                 | 139                 |                         |                           |                                  | 116                     |                       |
| L                 | 165                 |                         | 151                       | 147                              | 111                     | 100                   |
| LC                | 172                 |                         | 136                       | 139                              |                         | 59                    |
| LC                | 196                 |                         | 163                       | 228                              | 165                     | 136                   |
| L                 | 198                 | 210                     | 140                       | 140                              | 215                     | 148                   |
| IC                | 217                 | 257                     |                           |                                  |                         |                       |
| LC                | 218                 |                         | 160                       | 141                              |                         | 123                   |
| IC                | 220                 | 193                     |                           | 187                              |                         |                       |
| M                 | 221                 | 230                     | 115                       | 180                              | 164                     | 100                   |
| IC                | 281                 | 307                     |                           |                                  | 263                     |                       |
|                   |                     |                         |                           |                                  |                         |                       |
| I                 | 281                 | 383                     |                           | 174                              | 256                     |                       |
| MC                | 292                 | 380                     | 191                       | 188                              |                         | 122                   |
| I                 | 345                 | 360                     | 317                       | 241                              |                         | 136                   |
| I                 | 378                 | 427                     | 260                       | 241                              |                         | 151                   |
| $n^c$             | —                   | 32                      | 16                        | 23                               | 34                      | 16                    |
| $A^d$             | —                   | 24.2                    | -10.6                     | -55.5                            | 47.2                    | -48.8                 |
| $B^d$             | —                   | .79                     | 1.31                      | 1.44                             | .68                     | 2.21                  |
| $r^e$             | —                   | .97                     | .91                       | .71                              | .82                     | .73                   |
| $C_{100\%}^f$     | 5.1                 | .90                     | .45                       | .35                              | .95                     | .90                   |

<sup>a</sup>To simplify this comparison all proteins are expressed in an arbitrary unit defined as the percentage concentration ratio between the actual sample and a serum pool of healthy blood donors.

<sup>b</sup>Code: N = normals; Nb = newborns; L = low (or slight) inflammatory reaction; M = moderate degree of inflammatory reaction; I = intense inflammatory reaction; P = pregnant woman;  $\alpha$  = hemolytic disease (consumption of haptoglobin); C = chronic disease.

<sup>c</sup> $n$  = no. determinations.

<sup>d</sup> $\Delta A\% = A + Bx$ , where  $x$  is the concentration of the different proteins.

<sup>e</sup> $r$  = Correlation coefficient.

<sup>f</sup> $C_{100\%}$  is the actual concentration in g/liter corresponding to the serum pool used which is defined as being 100%. (For  $\Delta A\%$ : "Apparent albumin reactants" in g/liter is given).



Studies on the Slow Reaction Step

Figure 4 compares the earlier-defined  $\Delta A\%$  with values for  $\alpha_2$ -globulin obtained by cellulose acetate electrophoresis for sera from newborns, blood donors, and from patients for whom routine electrophoresis was done in the laboratory. The latter were classified by an independent interpreter. They were interpreted as being normal or showing slight, moderate, or intense acute inflammatory reaction on the basis of decrease in albumin and elevation of  $\alpha_1$ - and  $\alpha_2$ -globulin, or as chronic inflammatory reaction, based on elevation of  $\gamma$ -globulin.

We compared  $\Delta A\%$  for five proteins: orosomucoid, ceruloplasmin, complement C3(=  $\beta_{1A} - \beta_{1C}$ ), haptoglobin, and hemopexin. Haptoglobin was determined according to Tarukoski (22); the other proteins were evaluated by the Laurell "rocket" technique.

As Table 1 shows,  $\Delta A\%$  correlated well with orosomucoid and ceruloplasmin. These two proteins are accepted as acute phase reactants. Of the other proteins investigated, reasonable correlation exists between  $\Delta A\%$  and haptoglobin concentration, whereas the correlation with complement C3 and hemopexin is rather poor.

Table 1 includes the codes for different patient groups, to show some examples of the variations in  $\Delta A\%$  for these groups.

Discrepancies from the general pattern in Table 1 include the low haptoglobin values for newborns and for patients with hemolytic disease, and the high complement C3 values for newborns.

Factors Influencing the Reaction

*pH.* Table 2 shows the influence of pH (3.9 to 4.5) on the fast and slow reaction. Two patients' sera and one standard (40 g of albumin per liter) were tested. The sera were read against a blank solution of corresponding pH. The results for albumin and  $\Delta A\%$  were constant in this pH range, but there was a slight increase in the corresponding absorbances up to pH 4.4. Because all measurements were made with an appropriate blank solution, the influence of pH on the blank value is not considered here.

*Temperature.* The influence of temperature on the fast and slow reaction is shown in Table 3. The temperature was increased stepwise in the thermostatted cell. Two patients' sera (not the same as above) and one standard (40 g of albumin per liter) were tested. Sample and blank solutions were at the same temperature. The absorbance and final result for the fast reaction (albumin) was constant between 20 and 45 °C. For the slow reaction the absorbance differences increased with increasing temperature above 30 °C, but the results, expressed as  $\Delta A\%$ , were constant between 20 and 35 °C. An increase was noted for sample 1 at 40 and 45 °C. The second (slow) reaction showed a slightly increased reaction rate, presented in Table 3 as the absorbance difference between 1.5 and 0 min.

Information Obtained by the BCG Method

Figure 5 shows an example of a combination of the information from the fast (albumin) and the slow ( $\Delta A\%$ ) reaction step. Two sloping (dotted) lines were drawn to effect the best separation of the patient groups. The four solid lines represent the normal concentration range for albumin and  $\Delta A\%$ . The vertical and horizontal dotted lines separate different patient groups according to the intensity of their inflammatory reaction. Orosomucoid values indicate that the results represented by the four uppermost unfilled circles ("normal" patients from routine electrophoresis) should be designated "slight inflammatory response".

We could categorize the results for the different subjects into four groups: normals, newborns, and patients with slight and those with a more intense inflammatory reaction.

Discussion

We have demonstrated that normal and abnormal serum albumin concentrations can be reliably determined if the absorbance of the albumin/BCG complex is read at the time of mixing, 0 min. The experiments in which pure albumin standard or albumin prepared electrophoretically were used showed that the fast phase of the BCG reaction is attributable to albumin. By ex-

Table 2. Influence of pH on the Fast (Albumin) and the Slow ("Acute Phase Reactant") Phases of the BCG Reaction

| pH  | Albumin (0 min)     |                |                     |                |                     |                | $\Delta A\%$                   |                  |                                |                  |
|-----|---------------------|----------------|---------------------|----------------|---------------------|----------------|--------------------------------|------------------|--------------------------------|------------------|
|     | Standard            |                | Sample 1            |                | Sample 2            |                | Sample 1                       |                  | Sample 2                       |                  |
|     | A X 10 <sup>3</sup> | Concn, g/liter | A X 10 <sup>3</sup> | Concn, g/liter | A X 10 <sup>3</sup> | Concn, g/liter | $\Delta A^a$ X 10 <sup>3</sup> | $\Delta A\%$ , % | $\Delta A^a$ X 10 <sup>3</sup> | $\Delta A\%$ , % |
| 3.9 | 497                 | 40             | 507                 | 40.8           | 460                 | 37.0           | 080                            | 137              | 128                            | 220              |
| 4.0 | 515                 | 40             | 532                 | 41.3           | 483                 | 37.5           | 084                            | 139              | 137                            | 227              |
| 4.1 | 564                 | 40             | 579                 | 41.1           | 526                 | 37.3           | 091                            | 139              | 149                            | 227              |
| 4.2 | 609                 | 40             | 627                 | 41.2           | 564                 | 37.0           | 097                            | 137              | 158                            | 223              |
| 4.3 | 625                 | 40             | 641                 | 41.0           | 578                 | 37.0           | 101                            | 139              | 161                            | 220              |
| 4.4 | 631                 | 40             | 644                 | 40.8           | 579                 | 36.7           | 099                            | 135              | 162                            | 220              |
| 4.5 | 644                 | 40             | 621                 | 38.6           | 557                 | 34.6           | 105                            | 139              | 145                            | 193              |

<sup>a</sup>  $\Delta A = A(\text{at } 60 \text{ min}) - A(\text{at } 0 \text{ min})$ .



Table 3. Influence of Temperature on the Fast (Albumin) and the Slow ("Acute Phase Reactant") Phases of the BCG Reaction

| Temp,<br>°C | Albumin (0 min) |                   |                 |                   |                 |                   | $\Delta A\%$                        |                               |                   |                                     |                               |                   |
|-------------|-----------------|-------------------|-----------------|-------------------|-----------------|-------------------|-------------------------------------|-------------------------------|-------------------|-------------------------------------|-------------------------------|-------------------|
|             | Standard        |                   | Sample 1        |                   | Sample 2        |                   | Sample 1                            |                               |                   | Sample 2                            |                               |                   |
|             | $A \times 10^3$ | Concn,<br>g/liter | $A \times 10^3$ | Concn,<br>g/liter | $A \times 10^3$ | Concn,<br>g/liter | $\Delta A_{1.5}^a$<br>$\times 10^3$ | $\Delta A^b$<br>$\times 10^3$ | $\Delta A\%$<br>% | $\Delta A_{1.5}^a$<br>$\times 10^3$ | $\Delta A^b$<br>$\times 10^3$ | $\Delta A\%$<br>% |
| 20          | 602             | 40                | 579             | 38.5              | 715             | 47.5              | 017                                 | 084                           | 112               | 019                                 | 075                           | 100               |
| 25          | 600             | 40                | 585             | 38.7              | 717             | 47.5              | 021                                 | 087                           | 115               | 021                                 | 074                           | 98                |
| 30          | 600             | 40                | 574             | 38.3              | 709             | 47.3              | 035                                 | 084                           | 112               | 029                                 | 075                           | 100               |
| 35          | 601             | 40                | 571             | 38.0              | 715             | 47.6              | 054                                 | 103                           | 114               | 037                                 | 090                           | 100               |
| 40          | 602             | 40                | 575             | 38.2              | 712             | 47.3              | 072                                 | 148                           | 148               | 053                                 | 099                           | 99                |
| 45          | 602             | 40                | 583             | 38.7              | 710             | 47.2              | 077                                 | 170                           | 131               | 056                                 | 131                           | 101               |

<sup>a</sup>  $\Delta A_{1.5} = A(\text{at } 1.5 \text{ min}) - A(\text{at } 0 \text{ min})$  is included in the table to demonstrate the increase in reaction rate with increasing temperature.

<sup>b</sup>  $\Delta A = A(\text{at } 60 \text{ min}) - A(\text{at } 0 \text{ min})$ .

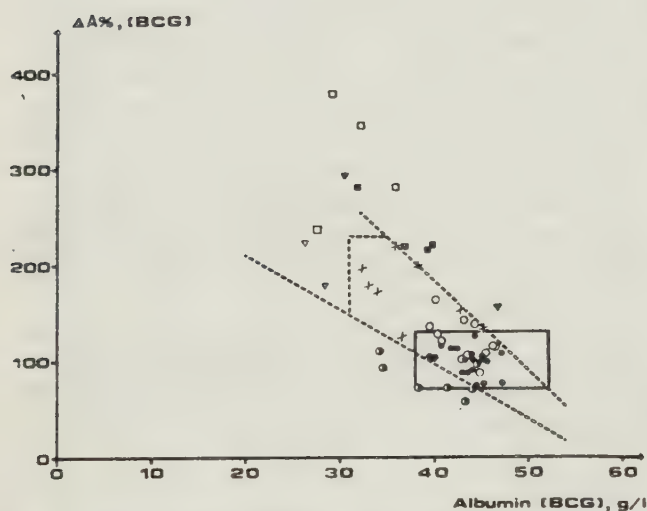


Fig. 5. Relation between values for albumin and  $\Delta A\%$ , obtained by the BCG method

Symbols same as in Figure 4. The four solid lines represent the normal concentration range for albumin and  $\Delta A\%$

trapolating the recorder registration to zero time, a reliable albumin value was obtained. As can be seen in Figure 3, results for the BCG method compare well with those for the Laurell "rocket" technique and, as shown above, with those for cellulose acetate electrophoresis. The use of two different statistical procedures gives a good correlation with no detectable systematic difference between the results from these method of albumin determination.

In contrast, values obtained with the BCG method after 60 min reaction time differ considerably from albumin values determined by more specific methods (Figure 3). Reaction times of 5 to 30 min have been used in all work published so far. The resulting values thus are higher than the true albumin values because the value includes other components, which react more slowly. As can be seen in Figure 1, most of the slow-reacting components react with BCG during the first 5 min, and after 30 min the slow reaction is complete.

As an example the albumin concentration in one sample was found to be 27 g/liter at 0 min, while at 60 min the value was 43 g/liter. The value by the "rocket" technique was 28.5 g/liter.

The problems discussed above arise especially in the lower concentration range, where the diagnostic and prognostic value of accurate albumin determinations is particularly great. The problem of the poor comparison of the BCG method with more specific methods have been shown, among others, by Webster et al. (15) and Schirardin and Ney (17).

Figure 2 shows an albumin standard curve as determined by the BCG method. It is linear up to about 75 g of albumin per liter, and it passes through the origin. Thus only one standard, 40 g of albumin per liter, is needed for routine analyses.

The results for albumin were unaffected by changing the reaction temperature between 20 and 45 °C. An increase in absorbance of the albumin/BCG complex was seen when the pH was increased from 3.9 to 4.5, but nevertheless the serum albumin values were constant between pH 3.9–4.4.

As defined above, the measure of the slow-reacting component is called  $\Delta A\%$ . In accordance with the practice in common use of expressing the concentration of proteins in serum, an arbitrary unit of  $\Delta A\%$  is defined as the ratio between the value for the actual sample and a pooled specimen of serum from apparently healthy blood donors. (This ratio is multiplied by 100 to give %.)

Others (15, 16) have shown that globulin fractions are responsible for the overestimation of albumin in sera with low albumin concentrations. We soon found that the contribution from the slow reaction varied considerably with sera from different patients.

Newborns showed low values (Figure 1) despite a high serum bilirubin content (35–70  $\mu\text{mol/liter}$ ). Serum samples from patients with intense inflammatory reaction patterns showed a very marked increase in the slow-reacting component, up to about 400% of the normal.

$\Delta A\%$  and the  $\alpha_2$ -globulin fraction from cellulose ac-



etate electrophoresis can be compared in Figure 4. The correlation is good and the patients are grouped along the regression line in order of increasing degree of inflammatory reaction. High concentrations of  $\gamma$ -globulin, characteristic of chronic inflammatory reaction, did not affect the results.

As Table 1 shows,  $\Delta A\%$  seems to be correlated to the intensity of the inflammatory reaction; we found a good correlation between  $\Delta A\%$  and orosomucoid and between  $\Delta A\%$  and ceruloplasmin.

The haptoglobin concentration determined, according to Tarukoski (22), was also compared with  $\Delta A\%$ . Discrepancies were found in the case of sera from newborns and patients with hemolytic disease. Haptoglobin measured by "rocket" immunoelectrophoresis showed essentially the same pattern except that newborns and patients with hemolytic disease showed even lower values.

Both complement C3 [we measured the inactive form,  $\beta_{1A}$  (23, 24) in most samples because the serum samples were not quite fresh] and hemopexin correlated poorly with  $\Delta A\%$ .

In a number of serum samples from pregnant women  $\Delta A\%$  was increased (130–170% of normal), suggesting that estrogen positively affects  $\Delta A\%$ .

We found a good correlation between the second (slow) reaction,  $\Delta A\%$ , and orosomucoid and ceruloplasmin values, which indicates that  $\Delta A\%$  can be used as a measure of "acute phase reactants," although we have not yet established which component(s) actually cause the slow reaction. This problem is currently being investigated.

Figure 5 shows the information from the two available measures of the BCG reaction, albumin and  $\Delta A\%$ . This diagram exemplifies how the two pieces of information obtained by this method can be combined. Although the lines in this figure are rather arbitrary and not intended to be definitive borderlines for different patient groups, it can be seen that it is possible to classify patients according to the degree of their inflammatory reaction. It is also apparent from this figure that attempts to calculate an "index" between albumin and  $\Delta A\%$  will result in loss of information, and the various patient groups will be intermixed.

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## URINARY ALBUMIN DETERMINATION BY THE IMMEDIATE BROMCRESOL GREEN METHOD

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### Summary

It has been demonstrated recently that the reaction of serum samples with bromcresol green (BCG) reagent proceeds in two steps. Albumin is responsible for the immediate reaction while other serum proteins produce the slow reaction.

In this paper the immediate BCG reaction has been used for the determination of urinary albumin concentration in patients with proteinuria by a slightly modified method with a primary pH adjustment of the urine and the use of a urine blank.

Comparison of the immediate BCG method ( $y$ ) with Laurell "rocket" technique ( $x$ ) gave the following equation:  $y = 17.2 + 1.006x$  ( $n = 98$ ;  $r = 0.99$ ) mg/l. The coefficient of variation (within-day), C.V. (%), ranged between 0.9 and 2.7% depending on the albumin concentration. It is thus possible to carry out rapid, accurate and precise albumin determinations in urine samples using this simple method.

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### Introduction

An increasing interest in the determination of specific proteins in urine has been noted lately since all "total protein" methods available now have certain drawbacks, such as different sensitivity to the different proteins, and also have precision problems.

The method for urinary albumin determination described here is designed to ascertain the albumin concentration for patients with proteinuria. We intend to use it together with serum albumin [1] to obtain the albumin clearance. We

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will also follow the changes of urinary albumin during pregnancy. The purpose was not to discover just slightly increased levels of albumin in urine. If this is considered to be most important, another dilution factor can easily be chosen.

This method is based on the immediate reaction between albumin and bromocresol green (BCG) as described earlier for serum [1]. BCG for urinary albumin was used by Rasanayagam et al. [2] and BenGershom [3]. Neither of these considered the influence of the slow reacting proteins nor did they recommend subtraction of blanks. BenGershom [3] found that colored urines presented special problems which are discussed here.

The main advantage of the BCG method is its simplicity and its suitability for automation. With the suggested improvements, immediate reading, adjustment of pH for some urines and subtraction of blanks, the BCG method also proves to be a reliable and accurate method for routine use. The measurement of the high molecular weight "slow reacting proteins" \*, may be of use for determining the degree of selectivity in glomerular proteinuria. This, however, is the subject of a separate study.

## Materials and methods

### *BCG method*

*Reagents.* The reagents used were those described by Doumas et al. [5] for the manual method, with consideration of the points given in refs. 1 and 4 \*\*. A blank reagent was also prepared omitting the bromocresol green.

Kabi protein standard (KABI AB, S-10425 Stockholm, Sweden) was used. This standard was diluted to contain 40 g/l. From this the working standard was prepared.

*Apparatus.* The same as in ref. 1.

*Procedure.* First centrifuge the urine samples. Then check the approximate albumin content with "Albustix" (Ames Co., Elkhart, Ind. 46514) and dilute the urines to achieve an albumin concentration of <4000 mg/l (<2000 mg/l for better precision). Check the pH of the patient's urine with an indicator strip. We used "Duotest" (Macherey-Nagel and Co., 516 Düren, G.F.R., P.O. Box 307) pH range 1–12. For urines with a pH above 6.5 an adjustment was made to pH 5.0 with 5 mol/l hydrochloric acid. The pH was checked with "Duotest" pH range 3.5–6.8. (This indicator paper gave, by its two color fields, a clear and accurate pH reading which agreed well with readings on a pH meter.)

Pipet 2.0 ml of dye solution into a reagent blank and a measuring cuvette. Add 200  $\mu$ l distilled water to the blank cuvette and 200  $\mu$ l of urine sample (diluted, pH adjusted or not) or standard into the measuring cuvette. Use a hand pipette (Eppendorf Gerätebau, Metheler and Hinz GmbH, 2000 Hamburg 63, G.F.R., Postfach 630 324) with disposable tips. Stir immediately. (Good mixing without introducing air bubbles was obtained by using an Eppendorf "Rühr-Stab" 3351.) Note the exact time of mixing. Follow the increase in

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\*\* Per liter: 0.15 mmol of BCG, 75 mmol of sodium succinate, and 4.0 ml of Brij-35 (300 g/l); pH 4.20  $\pm$  0.05.

absorbance for about 0.5 min on the recorder before the next sample is measured. (When the slow reacting proteins were investigated another absorbance measurement was made after 60 min at room temperature.) Extrapolate the curve back to the time of mixing.

The urinary blanks are obtained by mixing 200  $\mu$ l of the urine samples with the blank reagent. No timing is necessary for the blanks.

#### *Electroimmunoassay (EIA)*

The Laurell [6,7] "rocket" technique was used. Antihuman albumin anti-serum (Dakopatt A/S, DK 2900 Hellerup, Denmark) was added to a 10 g/l solution of buffered Indubiose A37 Agarose (L'Industrie Biologique Française S.A., Gennevilliers, Seine, France) at about 52°C.

Standards (as for the BCG method) and urine samples were diluted 20-fold with the electrophoresis buffer, a barbital/sodium barbital buffer (24 mmol/l, pH 8.6) [6,7].

5  $\mu$ l of the diluted samples was applied to each well and was electrophoresed at 12 V/cm for 3 h. The subsequent drying and staining procedures were carried out according to Axelsen et al. [7]. Peak heights were measured and the resulting standard curve was used to ascertain the albumin concentration in the samples.

## Results

#### *Choice of BCG reagent*

Three different reagent compositions were tried. The first was the same as used in ref. 1, the second had twice, while the third had four times the buffer concentration (75, 150 and 300 mmol sodium succinate per liter, respectively).

The standard curves for the three reagents can be seen in Fig. 1. The first curve had the steepest slope.

No significant difference could be shown between the three reagents for 11 patients' urine (Table I).

#### *Influence of the pH of patient urine*

Fig. 2 shows examples of titration curves obtained when adding dropwise 0.1 mol/l HCl or NaOH to different patients' urine. Most urines had a low buffer capacity but some urines, originally with a high pH, showed a more pronounced buffer capacity in the region of pH 5–8.

Fig. 3 shows the effect on the apparent "albumin" value when adjusting the pH of urine. Between pH 4.0 and pH 6.5 no significant effects of pH on urinary "albumin" values can be seen, while for some urines above 6.5 a large effect can be seen.

#### *The effect of ascorbic acid and Bence-Jones proteins*

No significant differences in albumin values could be detected for four patients' urine (containing 80, 160, 1160 and 3240 mg/l albumin) when ascorbic acid (final concentration: 0, 0, 56 and 2.7 mmol ascorbic acid/l urine) was added to each of these. Some of the urines in the comparison below con-

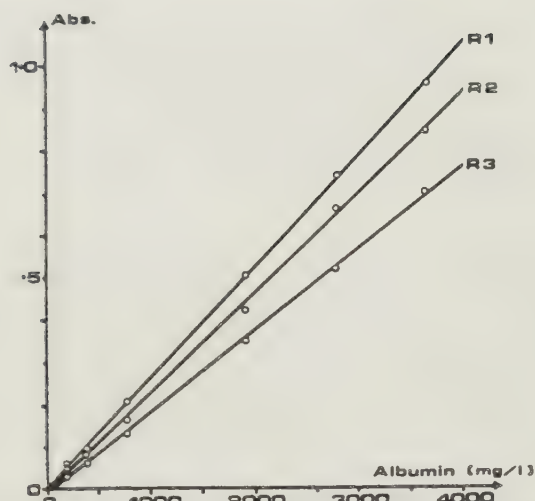


Fig. 1. Standard curves for three BCG reagents with different concentrations of succinate buffer (R1 = 75, R2 = 150, and R3 = 300 mmol/l).

tained large amounts of Bence-Jones proteins. No interference on the immediate BCG method could be shown.

#### *The BCG method compared with electroimmunoassay (EIA)*

Fig. 4 shows the comparison of urinary albumin values determined by the immediate BCG ( $y$ ) and EIA ( $x$ ) methods. The linear regression curve and correlation coefficient ( $r$ ) calculated by the Deming's procedure as described by Wakkers et al. [8] was:  $y = 17.2 + 1.006x$  ( $n = 98$ ;  $r = 0.99$ ).

Most of these urine samples were chosen with respect to certain pathological constituents in order to obtain urines with properties that could probably inter-

TABLE I

ALBUMIN RESULTS FROM 11 PATIENT URINES USING THREE BCG REAGENTS WITH DIFFERENT CONCENTRATIONS OF SUCCINATE BUFFER

BCG reagent: with succinate buffer R1 = 75, R2 = 150, R3 = 300 mmol/l.

| Albumin concentration (mg/l) |      |      |
|------------------------------|------|------|
| R1                           | R2   | R3   |
| 80                           | 100  | 80   |
| 140                          | 160  | 130  |
| 180                          | 220  | 140  |
| 240                          | 260  | 220  |
| 260                          | 260  | 220  |
| 260                          | 190  | 200  |
| 510                          | 510  | 460  |
| 1000                         | 910  | 920  |
| 1060                         | 1160 | 1280 |
| 3100                         | 3240 | 2900 |
| 3410                         | 3440 | 3460 |



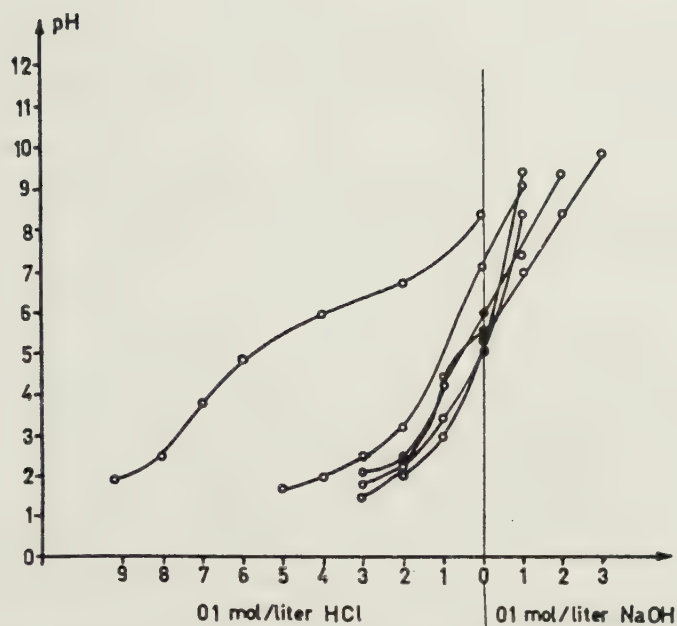


Fig. 2. Examples of "titration curves" for different patient's urine.

fere with the BCG method. They include urines with strong color (one greenish blue), and different levels of glucose, ketones, blood (hemoglobin), urea [9] or Bence-Jones proteins, some of which were substantially increased. Urines with high and low pH and different levels of proteins were also chosen. All

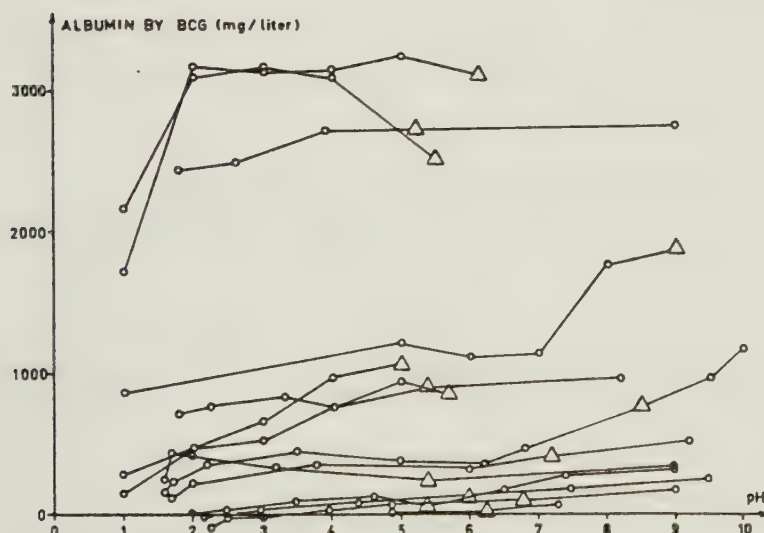


Fig. 3. The effect on the apparent "albumin" value when adjusting the pH of some patient's urine ( $\Delta$  indicates the original urinary pH,  $\circ$  are points obtained by adding hydrochloric acid or sodium hydroxide to the urines).

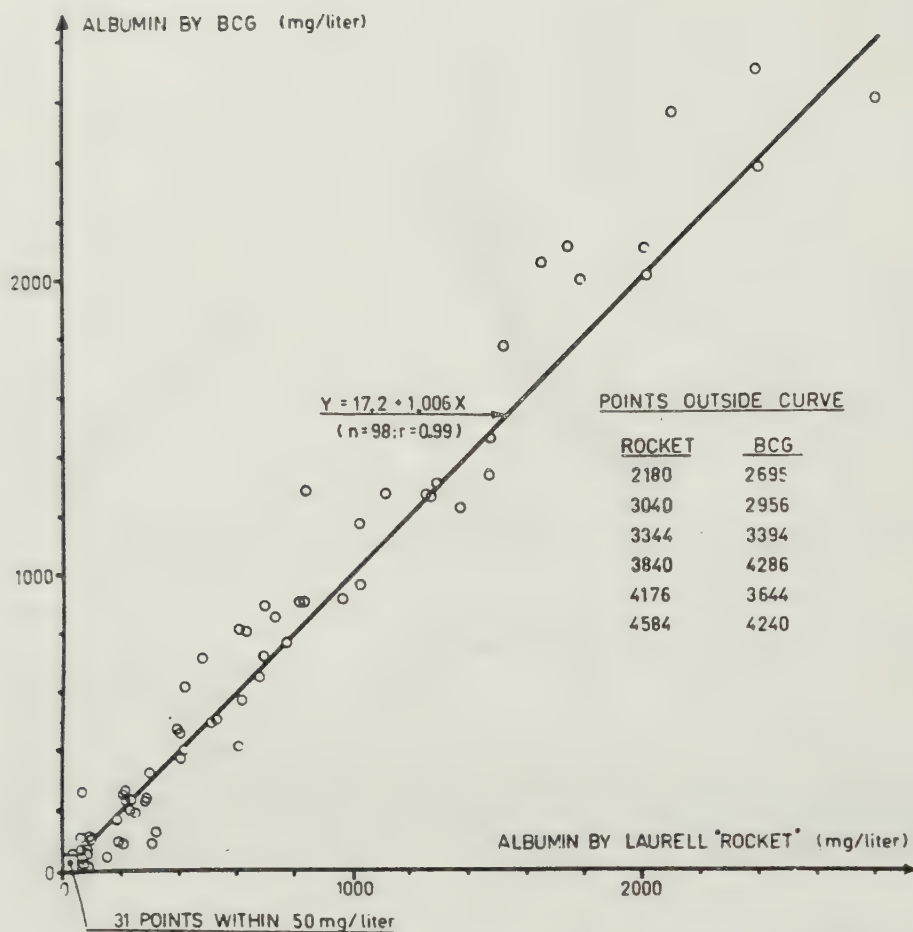


Fig. 4. Comparison of urinary albumin results as determined by the immediate BCG (y) and EIA (x) methods. Linear regression analysis, according to Deming's procedure as described by Wakkers et al. [8], was:  $y = 17.2 + 1.006x$  ( $n = 98$ ;  $r = 0.99$ ).

these BCG results are presented in Fig. 4 and in the comparison study.

The EIA (x) method was also compared with the BCG (y) results at 60 min. The linear regression equation was:  $y = 57.2 + 1.071x$  ( $n = 98$ ;  $r = 0.98$ ).

TABLE II

WITHIN-DAY PRECISION OF THE IMMEDIATE BCG METHOD FOR URINARY ALBUMIN DETERMINATION

20 replicates of standards at 3 levels.

| Mean<br>(mg/l) | C.V.<br>(%) | n  |
|----------------|-------------|----|
| 206            | 2.7         | 20 |
| 746            | 1.2         | 20 |
| 2640           | 0.9         | 20 |

### *Precision*

Table II shows the precision (within day) when running 20 standards at 3 levels.

### *Recovery*

Fig. 3 in ref. 9 shows the recovery obtained when albumin standard is added to urine samples. The range of the recovery was between 92% and 109%.

### *Discussion*

The immediate reaction [1] of albumin with BCG was used here to determine urinary albumin. The same reagent as for serum albumin determination was chosen for convenience. Since most urines with a high pH have a substantial buffer capacity (Fig. 2), the urines with pH above 6.5 were adjusted to 5.0. For some urines BenGershom [3] recommended adjusting the pH to 4.2. However, it was found earlier [4], that rearrangement of the slow reacting proteins occurred spontaneously at this pH and that they then react immediately, like albumin, when mixed with the BCG reagent.

The overall performance when using the immediate BCG method was only slightly better than the 60-min method (when comparing with EIA). Actually, most urines showed no significant difference. Nevertheless, in some urines, large differences were found ranging from a 50 to 100% false increase of the 60-min values.

This difference can most likely be used to ascertain the degree of selectivity for a glomerular proteinuria since the slow reacting acute phase protein in serum samples is a high molecular weight protein (see footnote \* in Introduction, p. 242). This will be investigated in a separate study.

It was found necessary to subtract urine blanks. Most urines have blank values below 10% of the actual albumin value but for colored samples it could be 20–30% (one greenish blue urine had a value of 40%).

BenGershom [3] suggested eliminating the effect of strong urine color by an adjustment of the pH to 4.2. He failed to correct for the original color of the urine by reading against a blank. We found that acceptable results were obtained with a blank correction, pH adjustment and an immediate reading, since colored urines often showed both a high pH and high levels of slow reacting proteins. When the urine blank is very high ( $>0.1$  A) an immunological method is recommended for precision work.

First we compared this immediate BCG method with the Mancini single radial immunodiffusion (SRID) technique. However, we found large differences between the two methods, SRID giving falsely high results. This will be presented in a separate paper [9] together with a solution to the problems in SRID.

The Laurell "rocket" technique (EIA) was then chosen for comparison purposes. In Fig. 4 and in the results, it is shown that the regression line between EIA and BCG is straight with a slope near 1 and a negligible intercept, and with a correlation coefficient of 0.99. We selected some normal urine samples but most samples in this comparison were abnormal in several respects, as shown in results above. This was done in order to investigate the susceptibility of the



BCG method to those variables. The precision of the method is satisfactory, ranging from 0.9% to 2.7%, depending on the albumin level.

The BCG method is thus reliable and specific. If a high throughput of samples is needed, automation of the method can easily be carried out along the guidelines given in ref. 4 by merely changing the dilution factor.

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*Clinica Chimica Acta*, 90 (1978) 249–257  
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## THE INFLUENCE OF CITRATE AND PHOSPHATE ON THE MANCINI SINGLE RADIAL IMMUNODIFFUSION TECHNIQUE AND SUGGESTED IMPROVEMENTS FOR THE DETERMINATION OF URINARY ALBUMIN

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### Summary

When adapting the immediate bromocresol green method for urinary albumin determination a correlation study with the Mancini single radial immunodiffusion (SRID) method was performed. This study showed large disparities between the two methods, SRID giving the higher results. The unsuitability of the currently used SRID methods is demonstrated and improvements to the method are suggested.

Known amounts of albumin were added to urine samples as well as to a "synthetic urine", both giving falsely elevated results with the SRID method. On investigating the different components of the "synthetic urine", it was found that the disparities were due to the influence of citrate and phosphate.

On addition of citric acid or phosphate to the dilution buffer and/or the gel buffer, the results of the SRID method agreed with those of other methods and with the expected values.

The findings presented in this paper can probably be extended to other immunological methods too since it seems to be the antigen-antibody reaction which is affected.

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### Introduction

The single radial immunodiffusion (SRID) method was originally described by Mancini et al. [1]. Since then several other papers have been presented in which the SRID method is used, among them the one by Becker [2]. This technique is a valuable tool in protein chemistry.

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SRID is commonly used for urinary albumin determinations. When studying the immediate bromcresol green (BCG) method [3] we found that the SRID method gave erroneously high results for albumin in urine [4], while both the Laurell "rocket" [5] technique (EIA) and the immediate BCG method gave the expected results.

It is shown in this paper why the SRID technique results in falsely elevated albumin results for urine samples. The methodological improvements suggested here should increase the reliability of the SRID technique in determining urinary albumin.

## Materials and methods

### SRID method

**Reagents.** A barbitol/sodium barbitol buffer 0.024 mmol/l, pH 8.6 [5] and Indubiose A37 Agarose (L'Industrie Biologique Française S.A., Gennevilliers, Seine, France) were used. The antihuman albumin antiserum came from Dako-patt A/S, DK 2900 Hellerup, Denmark. The addition of 7.0 mmol/l citric acid to the barbitol buffer is recommended.

**Methods.** The SRID procedure on 20-fold diluted samples was carried out as in ref. 1, but the drying and staining procedures as in ref. 6.

### Immediate BCG method

This method is performed according to Gustafsson and Uzqueda [4].

### "Synthetic urine"

The chemicals (analytical grade, Merck, Darmstadt, G.F.R.) were added and dissolved in the order shown below.

|                                                                      |           |    |
|----------------------------------------------------------------------|-----------|----|
| 1. Distilled water                                                   | about 500 | ml |
| 2. Potassium chloride (KCl)                                          | 3.80      | g  |
| 3. Sodium chloride (NaCl)                                            | 8.53      | g  |
| 4. Urea                                                              | 24.5      | g  |
| 5. Magnesium sulphate ( $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ ) | 1.03      | g  |
| 6. Citric acid                                                       | 1.03      | g  |
| 7. Ascorbic acid                                                     | 0.34      | g  |
| 8. Potassium phosphate ( $\text{K}_2\text{HPO}_4$ )                  | 1.18      | g  |
| 9. Creatinine                                                        | 1.40      | g  |
| 10. Sodium hydroxide (NaOH) (add slowly!)                            | 0.64      | g  |
| 11. Sodium bicarbonate ( $\text{NaHCO}_3$ )                          | 0.47      | g  |
| 12. Sulphuric acid ( $\text{H}_2\text{SO}_4$ ) (add slowly!)         | 0.496     | g  |

Transfer to a 1-l flask and dilute to volume with distilled water. The pH, density, and osmolality without any further corrections of the "synthetic urine" are shown in Table I.

### Standards

Kabi protein standard (KABI AB, S-104 25 Stockholm, Sweden) was used. It was diluted with distilled water and with several other solutions according to the results section.

TABLE I  
PROPERTIES OF THE "SYNTHETIC URINE"

|            |               |
|------------|---------------|
| pH         | 6.8           |
| Density    | 1.015 kg/l    |
| Osmolality | 847 mosmol/kg |

## Results

### *Comparison of SRID with the immediate BCG method*

In Fig. 1 the immediate BCG method ( $y$ ) is compared to the SRID technique ( $x$ ). The linear regression curve and correlation coefficient ( $r$ ), calculated by the Deming's procedure as described by Wakkers et al. [7] was:  $y = 50.9 + 0.819x$  ( $n = 53$ ;  $r = 0.99$ ).

### *Dilution of urines with high albumin content*

We diluted some urines which contained high concentrations of albumin.

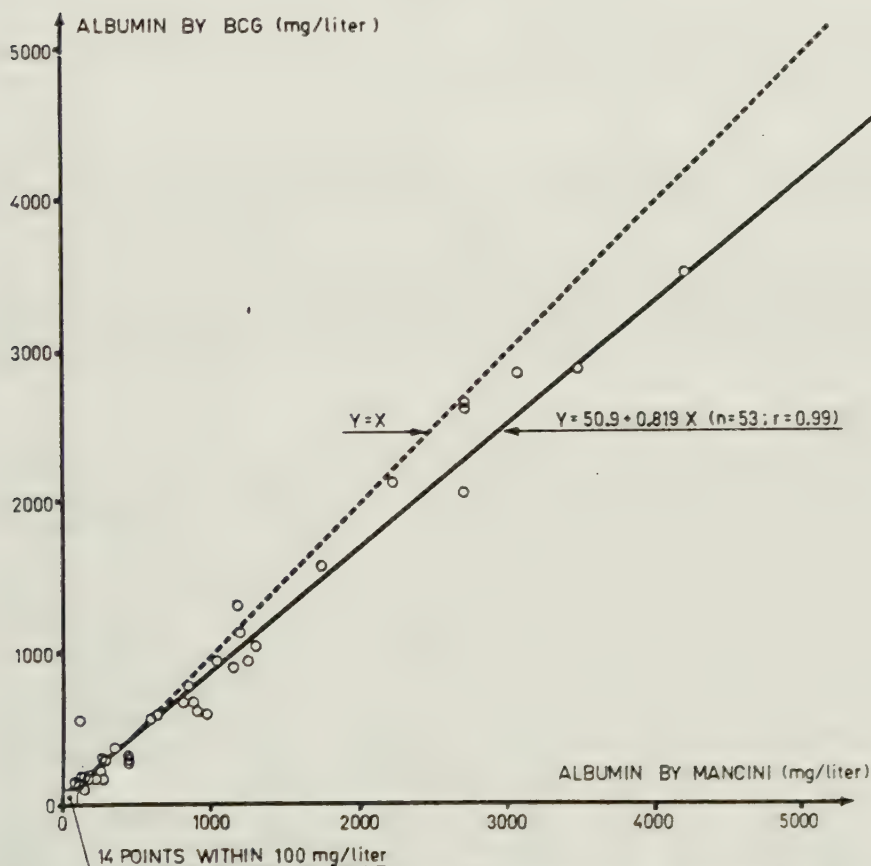


Fig. 1. Comparison of urinary albumin results as determined by the immediate BCG and Mancini radial immunodiffusion (SRID) methods. Linear regression analysis, according to Deming's procedure, as described by Wakkers et al. [7] was:  $y = 50.9 + 0.819x$  ( $n = 53$ ;  $r = 0.99$ ).



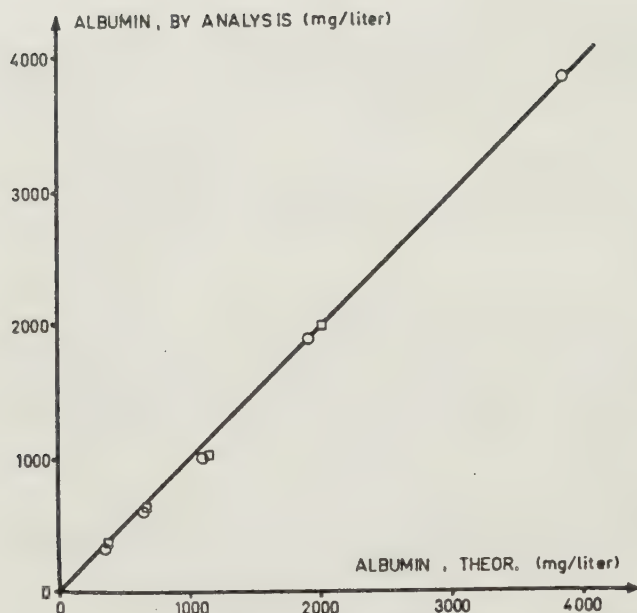


Fig. 2. Dilutions of urines with high albumin content. □, The immediate BCG method; ○, Mancini single radial immunodiffusion (SRID).

The expected results calculated from the dilution factors were obtained for both the BCG and the SRID methods on the same urine sample (Fig. 2).

#### *Addition of known amounts of albumin to urine samples*

Known amounts of albumin were added to several urines with no or with low albumin concentrations. As seen in Fig. 3 both the BCG method and EIA gave the expected results. However, the SRID results were falsely high.

#### *Searching for the cause of the problems with SRID*

**Urea.** Different amounts of urea (between 0 and 666 mmol/l in 6 steps) were added to standard solutions containing 1500 mg/l albumin. These were analysed for albumin by the SRID, "rocket" and BCG methods. No significant differences by the three methods could be shown.

**"Synthetic urine".** In order to obtain a simplified testing system, a "synthetic urine" was prepared as described above. The resulting pH, density, and osmolality are presented in Table I. The components added include 11 selected inorganic chemicals at levels contained in normal urines according to Free and Free, pp. 13–17 [8]. The analytical behaviour on adding albumin to the "synthetic urine" was the same as that in normal urines, i.e. BCG and EIA gave the expected values while SRID gave falsely elevated results.

**Components of "synthetic urine".** Nine of the chemicals added (sodium hydroxide and sulphuric acid were excluded) to the "synthetic urine" were investigated individually at the same final concentration as in the "synthetic urine". Different amounts of albumin were added to each of these nine solutions. Citrate had the most pronounced effect (Table II) on the SRID results

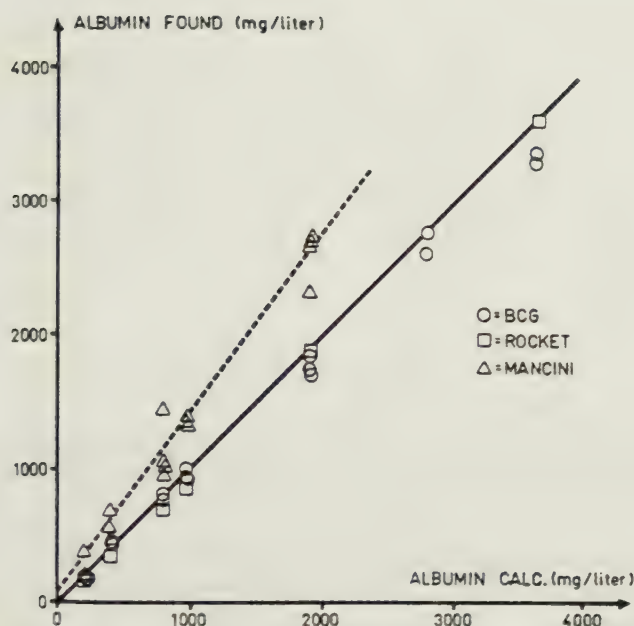


Fig. 3. Addition of known amounts of albumin to urine samples. O, The immediate BCG method; □, Laurell "rocket" technique; Δ, Mancini single radial immunodiffusion (SRID).

followed by phosphate. None of the other components had any significant effect.

*Osmolality.* 670 mmol/l sodium chloride and 220 mmol/l potassium chloride were dissolved in the barbitol buffer, giving an osmolality of 1870 mosmol/kg. This buffer was used to dilute the standards and to prepare the agarose gel. No influence could be shown, indicating that high osmolality as such does not affect the SRID results.

TABLE II

INVESTIGATION OF THE INFLUENCE ON THE SRID METHOD BY THE INDIVIDUAL COMPONENTS OF THE "SYNTHETIC URINE"

The concentration of each substance is the same as in the "synthetic urine".

| Substance tested         | Albumin as determined by SRID (mg/l) |     |      |      |      |      |
|--------------------------|--------------------------------------|-----|------|------|------|------|
| Albumin added:<br>(mg/l) | 199                                  | 396 | 784  | 1905 | 2790 | 3636 |
| Potassium chloride       | 230                                  | 400 | 780  | 1910 | 3040 | 3800 |
| Sodium chloride          | 230                                  | 400 | 780  | 1860 | 2790 | 3580 |
| Urea                     | 200                                  | 400 | 800  | 2080 | 2960 | 3640 |
| Magnesium sulphate       | 210                                  | 400 | 800  | 2060 | 2790 | 3800 |
| Citric acid              | 330                                  | 600 | 1240 | 2960 | 3600 | 5000 |
| Ascorbic acid            | 240                                  | 390 | 740  | 1930 | 2790 | 3636 |
| Phosphate                | 260                                  | 520 | 980  | 2300 | 3100 | 3820 |
| Creatinine               | 240                                  | 390 | 520  | 1930 | 2720 | 3580 |
| Sodium carbonate         | 240                                  | 340 | 720  | 1820 | 2640 | 3340 |

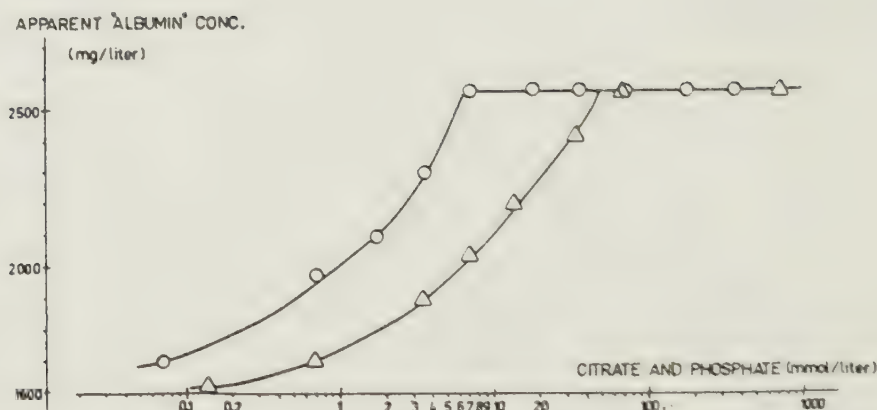


Fig. 4. The influence on the SRID method of different concentrations of citrate (○) and phosphate (△) in the buffer used. Logarithmic scale was used on the x-axis since a large concentration range was investigated.

*Barbital buffer 0.075 mol/l.* On using a more concentrated buffer solution [5] for running the SRID method, no improvement could be achieved when albumin was added to urine samples.

#### *Search for a remedy to the SRID problems*

*The influence of the concentration of citrate and phosphate.* Albumin was added to solutions of different concentrations of citrate or phosphate. These samples were run by SRID. The results can be seen in Fig. 4. Increasing concentrations of citrate or phosphate gave increased "albumin" values up to a certain level. At still higher concentrations no further effect could be detected. For citrate this upper level is 7.0 mmol/l and for phosphate 50 mmol/l.

*Influence of the pH of citrate and phosphate.* Hydrochloric acid or sodium hydroxide was added to citrate, 7.0 mmol/l, or phosphate, 13.6 mmol/l, solutions resulting in different pHs. Albumin was added to these solutions and they were run by SRID. No significant pH effects could be shown for citrate (pH 4.1 to 11.5 in 7 steps) or for phosphate (pH 3.9 to 11.3 in 10 steps).

*Addition of citric acid to the barbital buffer.* Albumin was added to distilled

TABLE III

#### ADDITION OF 7 mmol/l CITRIC ACID TO THE BARBITAL BUFFER

This buffer was used for dilution of all samples. For one of the investigations below it was also used for preparing the agarose gel, the other gel was prepared with an ordinary buffer.

| Type of plate                          | Type of sample    | Albumin added (mg/ml) | Albumin as determined by SRID (mg/l) |     |     |      |      |      |
|----------------------------------------|-------------------|-----------------------|--------------------------------------|-----|-----|------|------|------|
|                                        |                   |                       | 199                                  | 396 | 784 | 1905 | 2790 | 3636 |
| Agarose including citric acid 7 mmol/l | "synthetic urine" |                       | 170                                  | 420 | 820 | 2160 | 2790 | 3820 |
|                                        | Normal urine 1    |                       | 200                                  | 400 | 780 | 1960 | 2790 | 3640 |
|                                        | Normal urine 2    |                       | 200                                  | 410 | 780 | 1960 | 2790 | 3640 |
| Agarose without citric acid            | "synthetic urine" |                       | 160                                  | 400 | 780 | 1960 | 2820 | 3640 |
|                                        | Normal urine 1    |                       | 210                                  | 400 | 780 | 1910 | 2820 | 3820 |
|                                        | Normal urine 2    |                       | 210                                  | 400 | 820 | 1960 | 2900 | 3640 |



TABLE IV

CITRATE, PHOSPHATE AND BOTH ADDED TO THE DILUTION BUFFER AND TO THE STANDARDS

| Added mmol/l |           | Albumin found by SRID (mg/l) |            |
|--------------|-----------|------------------------------|------------|
| citrate      | phosphate | standard 1                   | standard 2 |
| 7            | —         | 1840                         | 3400       |
| —            | 14        | 1770                         | 3210       |
| 7            | 14        | 1890                         | 3400       |
| 14           | —         | 1870                         | 3500       |
| —            | 28        | 1890                         | 3140       |
| 14           | 28        | 1900                         | 3500       |

water, "synthetic urine" and two normal urines to obtain "standard curves" which were subjected to SRID analysis by two different procedures. In the first a buffer containing 7.0 mmol/l citrate was used both for sample dilution and agarose preparation but in the second only for sample dilution. No significant differences could be shown (Table III). The most convenient technique can thus be chosen.

Some differences in the appearance of the colored plates could, however, be seen, as will be described below.

*Effect by citrate and phosphate alone and in combination.* No significant difference between buffers containing either citrate or phosphate or both could be seen (Table IV). The effect is thus not additive for the two compounds.

*Color changes on the SRID plates.* When samples containing citrate or phosphate (including urine samples and "synthetic urine") were subjected to the SRID technique, a perfectly clear circular background around the precipitate rings could be seen against the normal faintly bluish background. This unstained area was also seen for citrate or phosphate without albumin. The clear area became greater or smaller depending on the concentration of these two compounds. We found (as in Fig. 4) that when the clear zone was larger (high citrate levels) than the precipitate ring, constantly elevated albumin results were found. As the clear zone reached the same size as the precipitate ring and became smaller than it, the resulting albumin values decreased, and finally reached their correct values. The different size of the precipitate rings was also clearly seen in unwashed and unstained plates. Thus, it is not merely a difference caused by the washing and staining procedure. On the plate with a gel prepared from the buffer with citric acid added, a perfectly clear background was obtained over the entire plate.

## Discussion

The comparison between the immediate BCG method and the SRID method was rather poor, SRID giving falsely elevated albumin values. The Laurell "rocket" technique (EIA) however showed good agreement with the immediate BCG method [4].

Dilution of the urines containing high levels of albumin gave the calculated dilution curves. However, by adding known amounts of albumin to urine sam-

ples with low albumin concentrations the nature of the problem became apparent.

The influence by urea was investigated first since it was earlier known to inhibit the antigen-antibody reaction. Here no such effect on SRID could be shown. Killingsworth and Savory [9] found this inhibition in a nephelometric method (0.2–1.2 mol/l urea in PBS gives 10 times the physiological concentration). They later [10] found no effect when diluting the samples 50-fold in PBS. They [9] may have obtained a partial influence by the phosphate buffer (10 mmol/l) (Table II and Fig. 4).

In order to obtain a simplified testing system we prepared a "synthetic urine" to use with the SRID method. It affected the SRID technique in the same way as normal urines.

When the different components of the "synthetic urine" were tested it was apparent that citric acid and phosphate influenced the SRID method. A "saturation effect" of the two compounds was found, and on the basis of this an improved SRID method could be designed. Thus, a barbitol buffer containing 7.0 mmol/l citrate is used to dilute the samples and to prepare the agarose gel. Accuracy is achieved and the method is suitable for routine use.

The addition of citrate to the agarose gel also results in better washing out properties of the antiserum from the gel on the SRID plates. A simpler and less time consuming washing procedure can thus be used.

It is also possible to use a phosphate buffer (>50–60 mmol/l). It is common (i.e. refs. 1 and 2) to use phosphate buffers with low concentration (<50 mmol/l) or other buffers, i.e. barbitol buffer. These are unsuitable for urinary albumin determinations by SRID if the method is not modified in any other way.

The enlarged precipitate rings and the color effects (results) indicate that the SRID problems with samples containing citrate and/or phosphate occur either indirectly by a binding to the immunoglobulins in the antiserum (the antiserum used contains only the immunoglobulin fraction) with a following change in its antibody binding properties, or directly by affecting the antigen-antibody reaction. It may also be an effect on the weak bindings between the immunoglobulins and the agarose gel.

These problems may also be seen when other urinary proteins are analysed by SRID or by other techniques (in which the citric acid or phosphate is left in the reaction mixture), for example, nephelometric methods. EIA is not affected, probably because these negatively charged small molecules move much faster than the proteins in the applied electric field and, therefore, cannot influence the antigen-antibody reaction.

This may also apply to other techniques for the determination of urinary proteins, such as hormone assays by RIA, EMIT, ELISA, and different pregnancy tests. The influence on the latter methods, as well as on nephelometric methods and SRID, for other proteins will be the subject of future studies.

Similar phenomena were recently described by Vader et al. [11] for the influence by other ions on radioimmunoassay. However, they use high concentration of these electrolytes, leading to the conclusion that this effect has a low practical importance. They did not investigate the effect by citrate and phosphate which we found to have a most pronounced effect in our system.

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# Automated Serum Albumin Determination by Use of the Immediate Reaction with Bromcresol Green Reagent

Jan E. C. Gustafsson

It has been demonstrated recently that the reaction of serum samples with bromcresol green reagent proceeds in two steps, albumin being responsible for the immediate reaction and other serum proteins for the slower one. I show here that the immediate reaction, used for albumin determination, can be automated. Two different automated systems are described: the LKB 2086 Reaction Rate Analyzer and the multichannel analysis system LKB 2480 Prisma. With the first system, 60 (or 120) samples per hour were run with a within-day standard deviation of 0.3 g/liter; with the second system, a feed rate of 150 samples per hour was used (SD, 0.3 g/liter, within-day). Comparison with other methods gave the following equations:  $y(\text{LKB 2086}) = 1.44 + 0.97x$  (immunodiffusion) ( $n = 43$ ;  $r = 0.98$ ) and  $y(\text{Prisma}) = 2.04 + 0.96x$  (manual method) ( $n = 73$ ;  $r = 0.98$ ). Thus albumin determinations can be done reliably, quickly, and simply at low reagent and labor cost.

The method for serum albumin determinations I describe here is based on the fast reaction (1) between albumin and an indicator dye, bromcresol green (BCG). Rodkey (2) first described the BCG method. Doumas et al. (3) improved it. The final reaction conditions used here are those described by Doumas et al., but the technique is improved.

Results by the BCG method did not compare well with more specific methods, when reaction times were prolonged. This was true especially in the clinically important lower concentration range (4, 5), where artificially high values were obtained for such sera. It was recently (1) shown that the reaction of serum samples with BCG proceeds in two steps. Albumin was shown to be responsible for the immediate reaction, while some other serum proteins contribute to a much slower reaction. A good comparison was found between the immediate albumin value and immunological methods, and these results have been confirmed by others (6, 7).

In a recent review by Peters (8) on serum albumin, he asked for the development of automated procedures based on this immediate reaction. In this paper two such automated systems are described.

The two analysis systems used are the LKB 2086 Reaction Rate Analyzer and the new multichannel analysis system LKB 2480 Prisma. To fit the manual method (1) to these two automatic systems, I used more concentrated reagents to initiate

the reaction between BCG and patients' sera after prediluting the serum samples with distilled water.

## Materials and Methods

### Reagents

*Reaction rate analyzer.* The BCG reagent used here is fivefold as concentrated as that described by Doumas et al. (3).

1. BCG Stock Solution; 3.0 mmol/liter: Dissolve 419 mg of bromcresol green (Hopkin & Williams Ltd, Romford RM1 1HA Essex, England) in 10.0 ml of 0.10 mol/liter sodium hydroxide solution in a stoppered tube. Mix overnight in an inverting rotating mixer. Transfer to a 200-ml volumetric flask and dilute to volume with distilled water.

2. Succinic Acid, Stock Solution; 500 mmol/liter: Dissolve 59.5 g of succinic acid in about 800–900 ml of water by magnetic stirring at room temperature overnight. Dilute to 1.00 liter with distilled water.

3. Brij-35, Stock Solution; 300 g/liter: Dissolve 30 g of the surfactant "Brij-35" (ICI America Inc., Atlas Chemical Division) in distilled water to a final volume of 100 ml by agitation with a magnetic stirrer overnight. This will result in a liquid with a high viscosity. (When using a commercial solution, check with your supplier that it really contains 300 g of Brij-35 per liter. At least one manufacturer has a "300 g/liter solution" containing only 230–240 g/liter Brij-35, which is of no importance in other applications, but should be avoided or corrected for here).

4. Working Dye Solution: Dilute 50 ml of BCG stock solution with 150 ml of succinic acid stock solution. Add 4.0 ml of Brij-35 stock solution. Adjust the pH to near 4.2 adding 5 mol/liter sodium hydroxide solution. The final adjustment should be made dropwise. After each drop the pH should be checked in a solution obtained by mixing 1.00 ml of working dye solution with 4.00 ml of distilled water. The pH of this diluted solution should then be  $4.20 \pm 0.05$ .

*Multichannel analysis system.* The concentration of the BCG working dye solution used is threefold that of the reagent used by Doumas et al. (3). The stock solutions are prepared as above.

*Working Dye Solution:* Dilute 50 ml of BCG stock solution with 150 ml of succinate buffer stock solution and add 130 ml of distilled water. Add 4.0 ml of Brij-35, 300 g/liter, stock solution. Adjust the pH as described above, except that 2.00 ml of working dye solution and 4.00 ml of distilled water are mixed for pH checking.

*Standard.* I used Kabi protein standard (KABI AB, S-104 25 Stockholm, Sweden). This standard is a 100 g/liter solution

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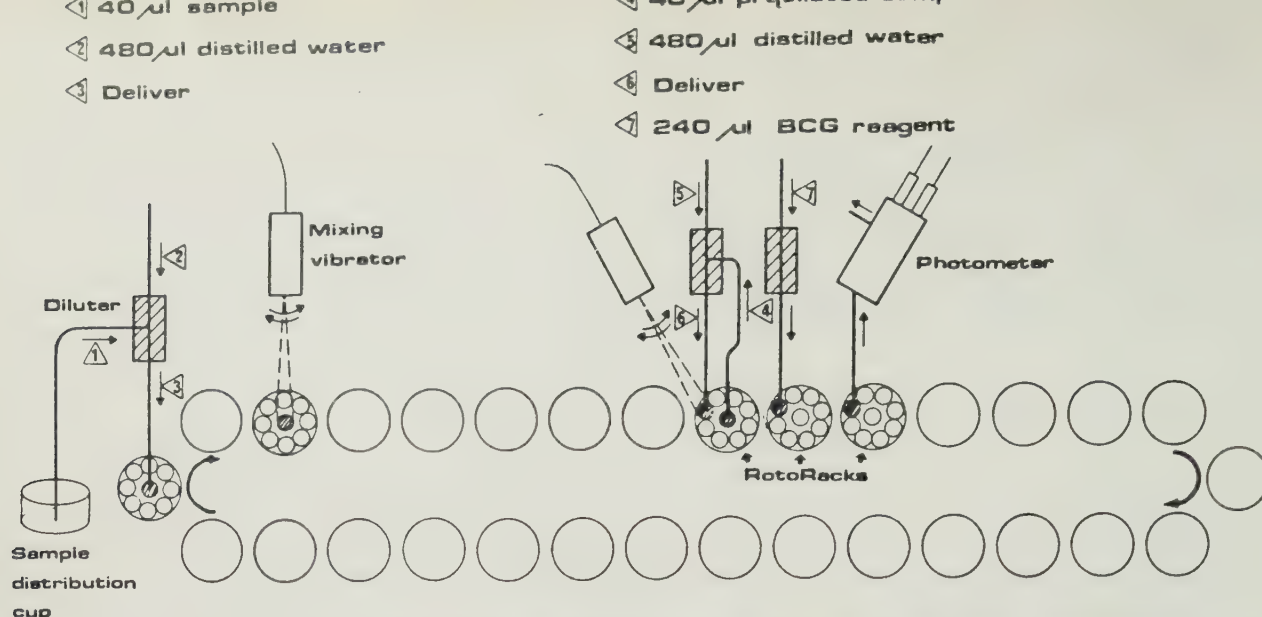


Fig. 1. Schematic drawing of a processing track (the BCG/albumin channel) from the multichannel analysis system LKB 2480 Prisma

The RotoRacks move clockwise around the continuous chain in 24-s intervals (150 samples/h)

of at least 98% pure human serum albumin, pH 7.3, containing no stabilizers or preservatives. It was diluted to contain 20, 40, and 60 g/liter.

**Control serum.** The control serum used was prepared by mixing serum samples from 100 apparently healthy blood donors. Aliquots from this serum pool were transferred to small tubes and frozen.

## Apparatus

The first system used was an LKB 2086 Reaction Rate Analyzer with an LKB 2076 Pneumatic Diluter/Dispenser (LKB-Produkter AB, S-161 25 Bromma 1, Sweden).

The second system used was the LKB 2480 Prisma, a new multichannel analysis system. This is a modular system with a central unit, processing modules, and support units. The maximum feed rate is 300 serum samples per hour, but we are now running the instrument at 150 samples per hour.

The sample tubes are loaded in racks. Aliquots of the samples are then transferred to the sample distribution cups, which move along the analyzer, passing the processing modules. When reaching one processing module, the required amount of sample for each analysis is, by means of a sampling loop, measured and transferred into one position of a Roto-Rack (Figure 1). In the RotoRack there are eight tubes, each of which can be used for one analysis. There is also one central tube which can be used for predilution of the samples. Each processing module has two processing tracks, individually thermostated. One track has 30 RotoRacks moving clockwise in a continuous chain. At different positions components can be placed for mixing, diluting, adding reagents, measuring the absorbance, and washing.

The different components and modules are supervised and controlled by a minicomputer. Another minicomputer is used for selecting the analysis to be performed on a serum sample, processing the measured values and presenting result reports and statistical information.

## Procedure

**Reaction Rate Analyzer.** With the automatic diluter add 35 µl of serum sample, standard, or control serum to 1000 µl of distilled water in small tubes. After mixing, dilute 35-µl

aliquots from these tubes with 1000 µl of distilled water into polystyrene cuvetts.

Use the following settings for the Reaction Rate Analyzer:

|                   |                         |
|-------------------|-------------------------|
| Wavelength        | 629 nm                  |
| Temperature       | 25, 30, 32, 35 or 37 °C |
| Light setting     | 0.1 A                   |
| Reaction course   | increase                |
| Injection nozzle  | black                   |
| Dispensing volume | 250 µl                  |
| Measuring time    | 1 min (or 30 s)         |
| Measuring range   | 0.2 A                   |
| Delay function    | —                       |
| Chart paper speed | 20 mm/min               |
| Mode of operation | hold background         |

First make the standard settings of the instrument, including the absorbance setting with distilled water. Then dispense 1000 µl of water into 10 cuvetts in a rack. Load the rack into the instrument. When the second cuvet has reached the measuring position, wait until a stable deflection of the recorder pen is obtained, and switch over to "hold background" operation. Check that the rest of the blanks are within acceptable limits. The hold-background function will now be stable for several hours.

The instrument is now prepared to run the standards and samples previously diluted. It is possible to follow the course of reaction on a recorder from about 3.2 s.

**Note:** The absorbance at 629 nm of the BCG reagent is very sensitive to temperature changes. Be sure to have enough B-reagent tubing in the thermostating block, because the volume of BCG-reagent is as high as 250 µl.

**Multichannel analysis system.** Standards, control serum, and serum samples are loaded in racks, which are placed on the loading table. Samples are then transferred into the sample-distribution system. An aliquot of the sample is transferred into the RotoRack as seen in Figure 1, where the following steps can also be seen. The RotoRack is located in a thermostated processing track in which the temperature is maintained at 25 °C. The absorbance is measured with a



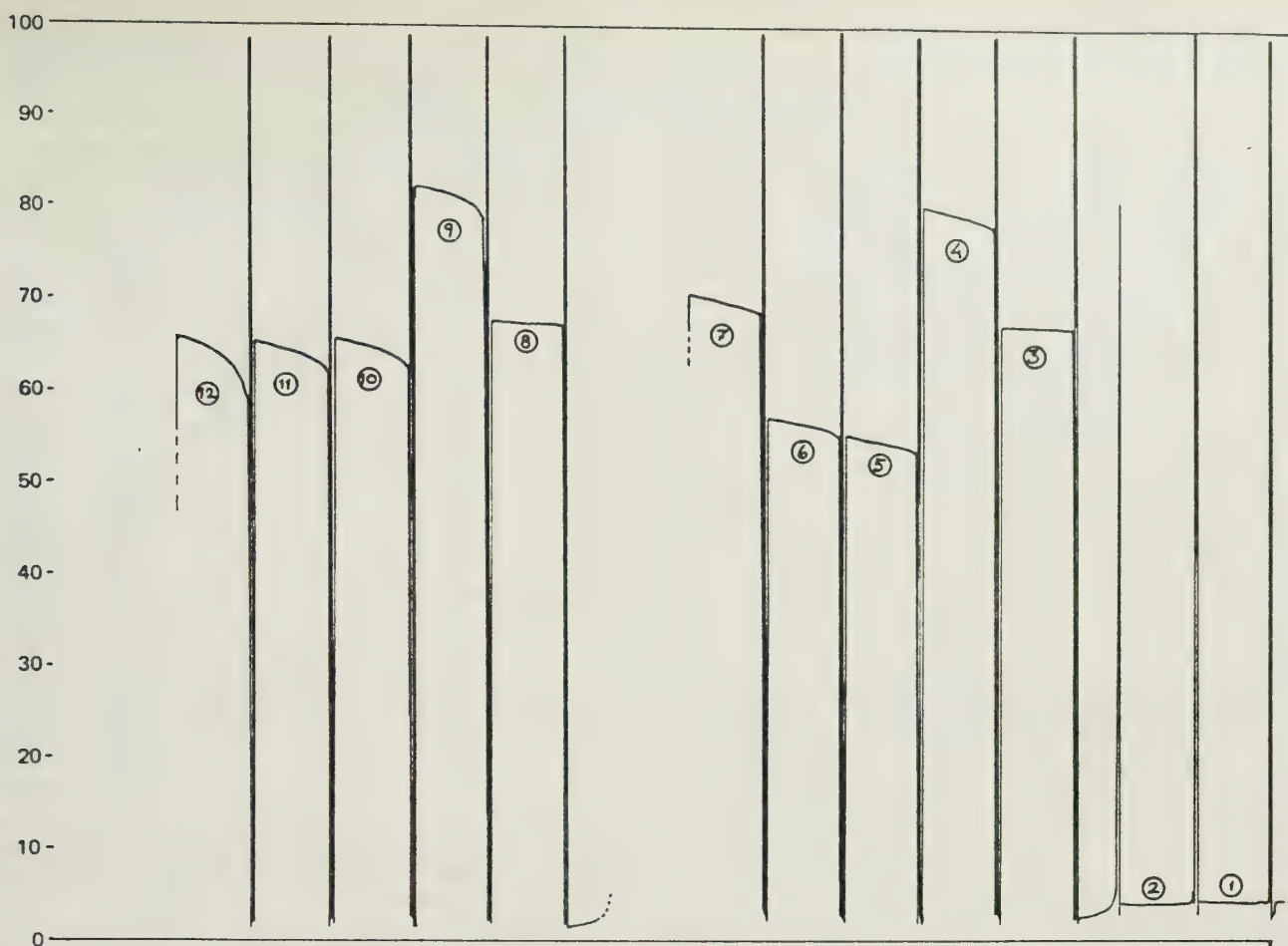


Fig. 2. Typical appearance of a recording on chart paper on using the LKB 2086 Reaction Rate Analyzer for albumin determination (1 min/sample)

1, 2: the final blank samples in the first rack; 3-7: instrument run at 25 °C; 8-12: instrument run at 37 °C; 3 & 8 are the standards; 4 & 9 are the control serum (serum pool); 5, 6, 7, 10, 11, and 12 are different patients' samples

photometer (629-nm interference filter) in the position next to where addition of starting reagent takes place.

## Calculations

**Reaction Rate Analyzer.** A typical chart-paper recording is seen in Figure 2.

The most nearly accurate results will be obtained if the line for each sample is extrapolated back to the moment of adding working dye solution. This can be done either manually, as seen in Figure 3, or automatically by a computer. The absorbance for each standard and serum sample is read. A standard curve is drawn and the concentration of the samples read from it. A simpler way is to use one standard to obtain a factor, which is then used to evaluate the samples. This is valid because the standard curve is linear and passes through the origin.

**Multichannel analysis system.** The absorbances obtained are automatically transferred into the minicomputer for evaluation. The results are calculated from one standard solution, since this system also gives a linear standard curve passing through the origin.

## Results

### Reaction Rate Analyzer

**Linearity and sensitivity.** The standard curve with albumin read immediately (extrapolated) after mixing reagent and standards is linear to above 60 g of albumin per liter, and passes through the origin. The dilution ratio used for the

samples is suitable for the instrument used, since it will give full recorder-pen deflection at an absorbance of 0.2 A (corresponding to about 65 g/liter albumin).

**Effect of reaction temperature.** A good comparison was obtained on comparing results at 25 °C (y) and 37 °C (x) (Figure 4), the regression equation being  $y = 0.17 + 1.00x$  ( $n = 47$ ;  $r = 0.98$ ) when using Deming's procedure (9). In Table 1 no significant differences can be seen in the results deter-

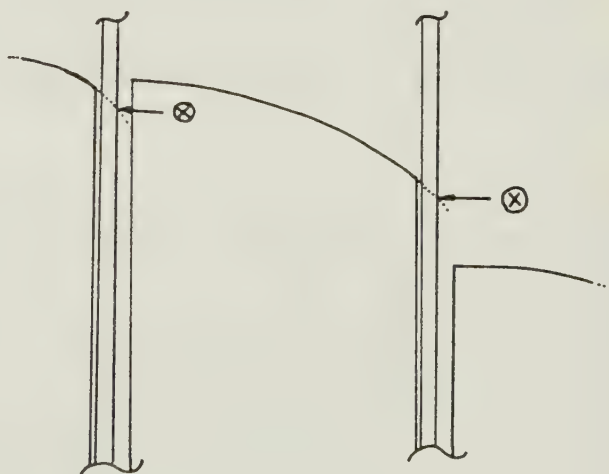


Fig. 3. Enlarged portion of the recording from Figure 2 to show the manual extrapolation

Read the absorbance at X



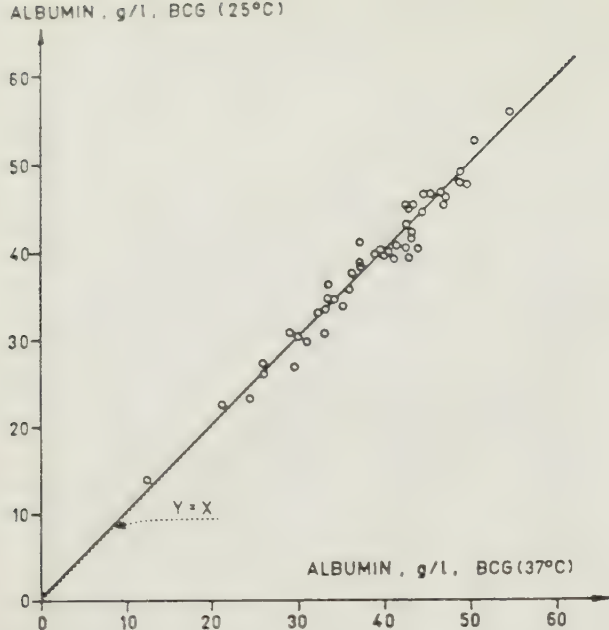


Fig. 4. Comparison of results at 25 °C with those at 37 °C for the immediate BCG method on the LKB 2086

$$y = 0.17 + 1.00x \quad (n = 47; r = 0.98)$$

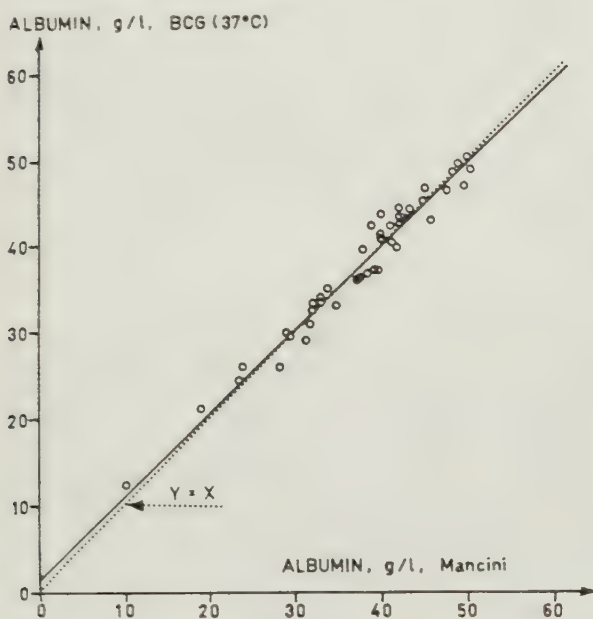


Fig. 5. Results by the immediate BCG method on the LKB 2086 at 37 °C as compared with results by Mancini radial immunodiffusion

$$y = 1.44 + 0.97x \quad (n = 43; r = 0.981)$$

mined at 25, 30, 35, or 37 °C. The Laurell "rocket" technique (electroimmunoassay, EIA) (10) and the manual method (1) have been described earlier.

**Comparison with Mancini radial immunodiffusion.** Figure 5 shows the comparison of results by the BCG method at 37 °C ( $y$ ) and Mancini radial immunodiffusion ( $x$ ) (11). The corresponding regression equation is given by:  $y = 1.44 + 0.97x$  ( $n = 43$ ;  $r = 0.981$ ), Deming's procedure (9).

**Precision.** The mean value for the control serum (serum pool from apparently healthy blood donors) was 46.7 g/liter. Within-run, 20 replicates showed a standard deviation of 0.3 g/liter. Day-to-day precision for the control serum, 15 different runs, was reflected in a standard deviation of 1.0 g/liter. On running duplicate analyses of 162 patients' sera (on several

Table 1. Comparison of Results for Albumin as Determined by the Laurell "Rocket" Technique (EIA), Manual BCG, and BCG on the Reaction Rate Analyzer at Different Temperatures

| EIA  | Manual BCG<br>g/liter | BCG on the LKB 2086, g/liter at: |       |       |       |
|------|-----------------------|----------------------------------|-------|-------|-------|
|      |                       | 25 °C                            | 30 °C | 35 °C | 37 °C |
| 8.6  | 9.4                   | 10.1                             | 10.3  | 11.2  | 11.1  |
| 21.4 | 22.0                  | 20.0                             | 20.6  | 20.8  | 21.4  |
| 23.0 | 22.3                  | 19.1                             | 19.9  | 20.1  | 20.0  |
| 24.5 | 24.3                  | 20.0                             | 20.2  | 20.4  | 20.1  |
| 28.4 | 28.0                  | 28.9                             | 28.9  | 29.1  | 29.9  |
| 29.6 | 31.0                  | 29.4                             | 29.1  | 29.6  | 31.7  |
| 30.3 | 31.1                  | 32.3                             | 31.6  | 32.6  | 32.2  |
| 32.3 | 33.4                  | 33.6                             | 34.1  | 33.9  | 33.7  |
| —    | 32.2                  | 32.2                             | 34.7  | 34.5  | 33.7  |
| 35.9 | 38.0                  | 36.6                             | 35.5  | 36.2  | 36.2  |
| 36.6 | 40.0                  | 40.1                             | 39.9  | 39.7  | 40.2  |
| 38.1 | 40.1                  | 36.6                             | 36.9  | 37.2  | 37.5  |
| —    | 40.0                  | 39.2                             | 40.1  | 40.2  | 39.5  |
| 42.4 | 43.0                  | 42.6                             | 42.7  | 43.0  | 42.4  |
| 44.0 | 47.0                  | 46.2                             | 45.7  | 46.0  | 46.6  |
| 47.8 | 47.0                  | 47.7                             | 48.4  | 48.8  | 48.4  |
| 47.8 | 46.0                  | 48.5                             | 49.5  | 49.7  | 49.4  |

days) the standard deviation of the difference between duplicates was 0.74 g/liter.

### Multichannel Analysis System

**Linearity.** The standard curve is linear to at least 60 g/liter and passes through the origin.

**Comparison with the manual BCG method described earlier (1).** When results from the multichannel analysis system ( $y$ ) and the manual method ( $x$ ) were compared, the regression analysis gave the following data:  $y = 2.04 + 0.96x$  ( $n = 73$ ;  $r = 0.97$ ) according to Deming's procedure (9) (Figure 6). These results were obtained from six different runs during January–March 1977.

**Precision.** When a control serum was analyzed on 21 different days, 50 to 100 samples per day, the within-day standard deviation ranged from 0.21 to 0.58 g/liter, a typical figure being about 0.3 g/liter.

### Discussion

The reaction between albumin and bromocresol green has been shown to be almost immediate (1). However, there are no commercial instruments now available that are suitable for routine use and that can measure the absorbance immediately after undiluted serum samples are mixed with reagent. It is thus necessary to investigate other possible solutions to this problem. One can either extrapolate the curve back to the time of mixing serum with reagent or can minimize the time until there are no significant errors in the results. Using the second method, I soon found that acceptable results were obtained when reaction times of less than 8–10 s were used [Figure 1 in (1)], as was also found by Corcoran and Durnan (7) to be an acceptable limit.

Another important factor in this method is the diluent used in the predilution step. Different diluents have been tried. Distilled water was best because use of either sodium chloride solution, 9 g/liter, or the succinate buffer used in the reagent gave erroneously high albumin values. There probably is more or less a conformational rearrangement of one or more of the slow-reacting proteins in the diluted samples. The rearranged

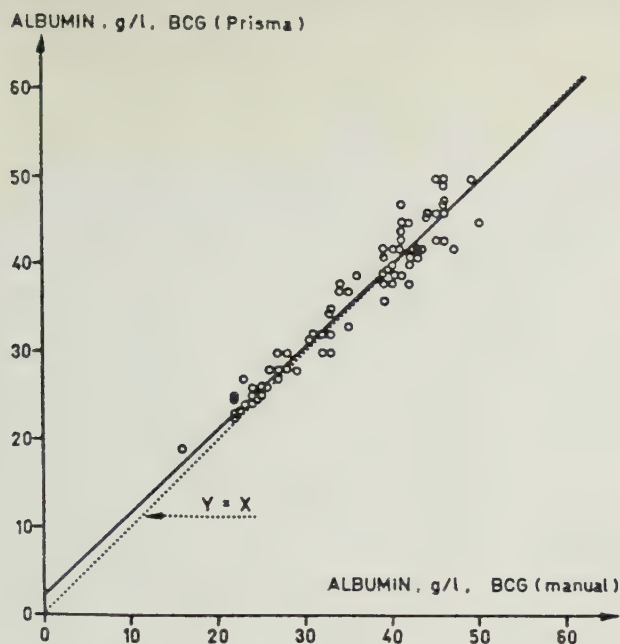


Fig. 6. Results by the immediate BCG method on the LKB 2480 Prisma as compared with results by the manual immediate BCG method (7)

$$y = 2.04 + 0.96x \quad (n = 73; r = 0.97)$$

protein will then react immediately with BCG reagent as if it were albumin. However it is possible that the high concentrations of immunoglobulins in some sera will cause a precipitation when diluted with water. This error is negligible because a very high dilution of serum is used in the final reaction mixture. Compare this with the results of Dumas (3) and Schirardin and Ney (5) for lipemic sera. The results presented by Webster (6), with a constant positive error of 3.0 g/liter, can perhaps be explained partly by the 20–30 s reaction time and partly by the predilution step in saline solution.

As shown in ref. 1, the rate of the slow reaction increases with increasing temperature. Thus the results most different from those by immunological methods would be expected to be those determined at 37 °C for sera with high levels of “acute phase reactants,” but it is shown here that good results were also obtained under these conditions. It is important to keep the system thermostated because the absorbance of the BCG reagent (blank) is sensitive to temperature changes. Higher temperatures give increasing absorbance. This is not the case for the albumin–BCG complex (1).

With either system I used, linear standard curves passing through the origin were obtained. This facilitated the use of only one standard, for example, 40 g/liter, in our routine

methods. This standard is instead run several times in a batch of samples in order to provide a reliable factor for evaluating the patient samples.

Albumin values by the Mancini radial immunodiffusion and the BCG method on the reaction rate analyzer compare well, as also is true for the comparison of our manual BCG method [earlier shown to have a good specificity for albumin (1)] and the BCG method with the Prisma multichannel analysis system. The latter results will be further improved on using shorter reaction times.

Regarding the points above, it should also be possible to adapt the immediate BCG method to other automated analysis systems.

It is shown that albumin determinations by the immediate BCG reaction can be carried out by different automatic analysis systems with a good comparison with immunological methods. Thus it is now possible to reliably determine albumin automatically at a low reagent and labor cost by a fast, simple method.

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Albumin Determination in Cerebrospinal Fluid by a Manual and an  
Automated Immediate Bromcresol Green Method

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### Summary

The immediate bromocresol green (BCG) method have earlier been used for determination of albumin in serum and urine.

In this study this method have been adopted to albumin determination in Cerebrospinal fluid (CSF). A manual as well as an automated procedure using LKB 2086 Reaction Rate Analyzer is described. Comparison of the manual immediate BCG method (y) with Laurell "rocket" technique (x) gave the following equation:  $y = 16.9 + 0.995x$  (n=51; r=0.995) mg/l. The coefficient of variation was 2.4% at 173 mg/l and 0.6% at 516 mg/l. Comparison of the automated (y) and the manual (x) immediate BCG methods gave:  $y = 1.9 + 0.996x$  (n=16; r=0.970) mg/l.

It is thus possible to carry out rapid, accurate and precise albumin determinations in CSF samples using this simple method.

### Key-Words

Albumin, Cerebrospinal Fluid, BCG immediate, Automated, Electro-immuno assay, Deming's procedure.

## Materials and Methods

### Human Cerebrospinal Fluid Samples

Lumbar spinal fluid samples were randomly collected, from the clinical chemical laboratory, after that the routine tests have been performed on them, i.e. electrophoresis, total protein determination.

### Manual BCG Method

Reagents. First prepare the "working dye solution" according to (5) for the Reaction Rate Analyzer. Dilute 15 ml of this solution with distilled water to 50 ml. This diluted BCG solution is the working BCG solution for this method.

Kabi protein standard (KABI AB, S-10425 Stockholm, Sweden) was used. This standard was diluted, by weighing, to contain 50 g/l. From this standard the working standards were prepared by further dilution to contain 50, 100, 250, 500, 750 and 1000 mg/l albumin.

Apparatus. The same as in ref. (3).

Procedure. Pipet 1.50 ml of dye solution into a reagent blank and into measuring cuvettes. Add 500  $\mu$ l distilled water to the blank cuvette. Put the cuvettes in the spectrophotometer for thermostating the solutions to 25°C. Keep standards, CSF samples and working BCG solution thermostatted at 25°C. Add then 500  $\mu$ l standard or CSF sample into the measuring cuvette. Use a hand pipette (Eppendorf Gerätebau, Metheler+Hinz GmbH, 2000 Hamburg 63, Postfach 630324) with disposable tips. Stir immediately (good mixing without introducing air bubbles was obtained by using an Eppendorf "Rührstab" 3351). Note the exact time of mixing. Follow the increase in absorbance for about 0.5 min on the recorder before the next sample is measured. Extrapolate the curve back to the time of mixing. Draw the standard curve and read the concentration of the samples from it.

## Introduction

Increased protein content in the cerebrospinal fluid (CSF) usually reflects an increased passage of plasma proteins through a damaged blood-CSF barrier, or a liberation of proteins from the central nervous system. A blood-CSF barrier damage gives an almost uniform increase of serum proteins of identical molecular radius. Local protein production or degradation gives an increase of selective proteins.

Ganrot and Laurell (1) compared the CSF/plasma ratios for albumin and IgG graphically. Olsson and Pettersson (2) used the IgG/Albumin index, calculated as the quotient of the CSF/serum ratios of IgG and albumin. Both papers describe the clinical usefulness of these parameters, in (2) combined with electrophoresis, for the diagnosis of multiple sclerosis (MS). Albumin was used for the quotient in preference for total protein (1).

The earlier described immediate BCG method for serum albumin (3), based on the method by Doumas et al. (4), together with the immediate BCG method for albumin in CSF described in this paper, can be used for determining the CSF/plasma ratio of albumin. This ratio can be used as such for the determination of the severity of a blood-CSF barrier damage or can be used together with IgG determination for calculating the IgG/Albumin index.

Two immediate BCG methods are described, one manual method and one automated method using the LKB 2086 Reaction Rate Analyzer.



Automated BCG Method

Reagents. The same as the "working dye solution" according to (5) for the Reaction Rate Analyzer.

The same working albumin standards as described for the manual BCG method.

Apparatus. The system used was the LKB 2086 Reaction Rate Analyzer (LKB-Produkter AB, S-16125 Bromma 1, Sweden) and an automatic diluter.

Procedure. With the automatic diluter, add 50  $\mu$ l working standard or CSF sample to 750  $\mu$ l distilled water into polystyrene cuvettes.

Use the following settings for the Reaction Rate Analyzer:

|                   |                 |
|-------------------|-----------------|
| Wavelength        | 629 nm          |
| Temperature       | 25°C            |
| Light setting     | 0.1 A           |
| Reaction course   | increase        |
| Injection nozzle  | black           |
| Dispensing volume | 200 $\mu$ l     |
| Measuring time    | 1 min (or 30s)  |
| Measuring range   | 0.2 A           |
| Delay function    | -               |
| Chart paper speed | 20 mm/min       |
| Mode of operation | hold background |

A detailed procedure can be seen in (5). 200  $\mu$ l working dye solution, is here used as B-reagent. Use 800  $\mu$ l instead of 1000  $\mu$ l distilled water for setting hold background.

The calculations are also described in (5).

### Electroimmunoassay (EIA)

The Laurell (6,7) "rocket" technique was used. Details on antiserum, agarose and procedure used is described in (8). The standards and CSF samples were diluted 10-fold.

### Results

#### Recovery for the Manual Immediate BCG Method and EIA

Figure 1 shows the recovery obtained when adding albumin standard to a CSF sample. The recoveries for the manual BCG method were 103, 96 and 100% when 250, 495 and 980 mg/l standard was added. The EIA gave 88, 95 and 100% recovery for the same samples.

#### Precision for the Manual Immediate BCG Method

Table I shows the precision (within-day) when running 20 standards at 2 concentration levels.

#### Dilution of CSF Samples with High Albumin Content

We diluted 2 CSF samples which contained high concentrations of albumin.

The expected results calculated from the dilution factors were obtained by the manual BCG method (Figure 2).

#### The Manual Immediate BCG Method Compared with Electroimmunoassay (EIA)

Figure 3 shows the comparison of CSF albumin values determined by the manual immediate BCG method (y) and EIA (x). The linear regression curve and correlation coefficient (r) calculated by the Deming's procedure as described by Wakkers et al. (9) was:

$$Y = 16.9 + 0.995x \quad (n=51; r=0.995).$$

### The Automated Immediate BCG Method Compared with the Manual BCG Method

Figure 4 shows the comparison of CSF albumin values determined by the automated (y) and manual (x) immediate BCG method.

The comparison as above was:

$$y = 1.9 + 0.996x \quad (n=16; r=0.970).$$

### Discussion

The immediate reaction (3) of albumin with BCG was used here to determine albumin in CSF. The recovery of added albumin standard to a CSF sample and the dilution series of two CSF samples, with a high albumin content, gave the expected results. These results together with an excellent agreement with the electroimmunoassay (EIA) indicate that the described immediate BCG method is specific for albumin and has a good accuracy. The precision is also good, the variation coefficient (CV%) is about 1/3 to 1/10 of the CV% for EIA depending on albumin concentration in CSF.

As an example of automation of the described method, we applied it on the LKB 2086 Reaction Rate Analyzer with a satisfactory result. With slight modifications of earlier published results for albumin determinations in serum (10-14), using the immediate BCG reaction (3), the following equipment can also be used: Technicon SMA 12/60 or AutoAnalyzer II, Plesher (10), Technicon SMAC, Cederblad et al. (11), Centrifugal Analyzer, Savory et al. (12) and King et al. (13), Flow-Injection analysis, Renoe et al. (14).

CSF albumin determination is a better choice than total protein determination since values for CSF albumin vary less between labora-



tories than values for total proteins, because methods and standards for calibration differ less from one laboratory to another (1). Albumin is principally produced by the liver why its concentration gives some information about changes in the permeability of the blood-CSF barrier and CSF turnover. Since albumin in plasma and in CSF are in equilibrium (after 5 days), the ratio of CSF albumin to plasma albumin will reflect the permeability and CSF formation slightly better than CSF albumin itself in nonacute changes of plasma albumin (1). This ratio can preferably be obtained by the immediate BCG method used for serum (3) and CSF.

This ratio between plasma and CSF albumin can also, together with IgG determination, be used for calculating the IgG/Albumin index, used for determination of local IgG production in or near the subarachnoid space.

The advantages of the immediate BCG methods, for determination of serum and CSF albumin, over immunological methods are their simplicity, reproducibility, low cost and suitability for automation. They are also shown to be specific for albumin.

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Table I

Within day precision of the manual immediate BCG method for albumin determination in Cerebrospinal Fluid.

20 replicates of standards at 2 levels.

| Mean<br>(mg/l) | C.V.<br>(%) | n  |
|----------------|-------------|----|
| 173            | 2.4         | 20 |
| 516            | 0.6         | 20 |

### Figure Legends

Fig. 1. The recovery obtained by addition of known amounts of albumin to a CSF sample

○ , The immediate BCG method

△ , Electroimmuno Assay (EIA)

Fig. 2. Dilution of CSF samples with a high albumin content. The manual immediate BCG method was used.

○ , CSF sample No 1

△ , CSF sample No 2.

Fig. 3. Comparison of CSF albumin results as determined by the manual immediate BCG method and electroimmuno assay. Linear regression analysis, according to Deming's procedure, as described by Wakkers et al. (9) was :

$$y = 16.9 + 0.995x \quad (n=51; r=0.995).$$

Fig. 4. Comparison of CSF albumin as determined by the automated and manual immediate BCG methods. Linear regression analysis, according to Deming's procedure, as described by Wakkers et al. (9) was:

$$y = 1.9 + 0.996x \quad (n=16; r=0.970).$$

FIGURE 1

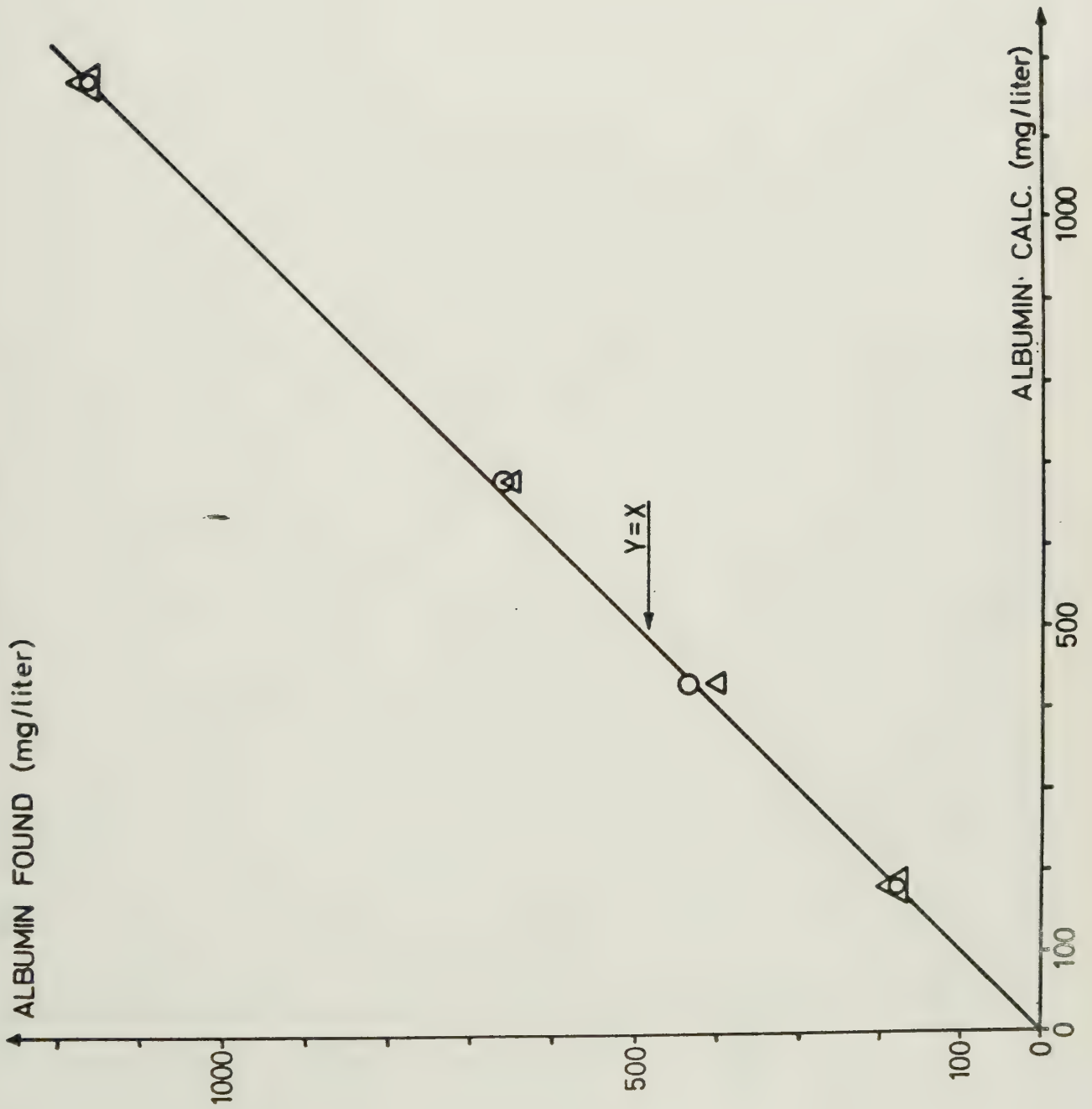




FIGURE 2

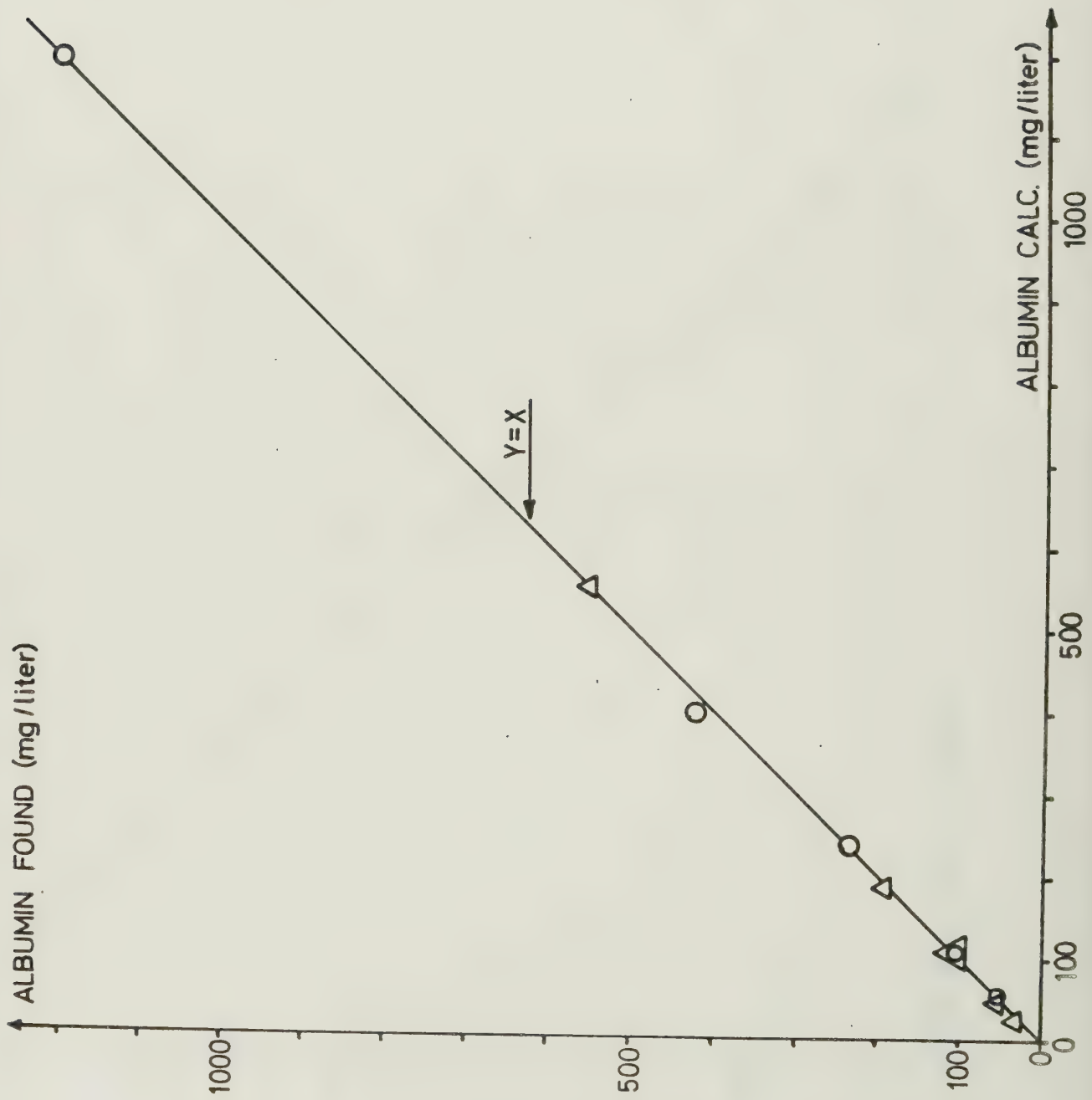


FIGURE 3

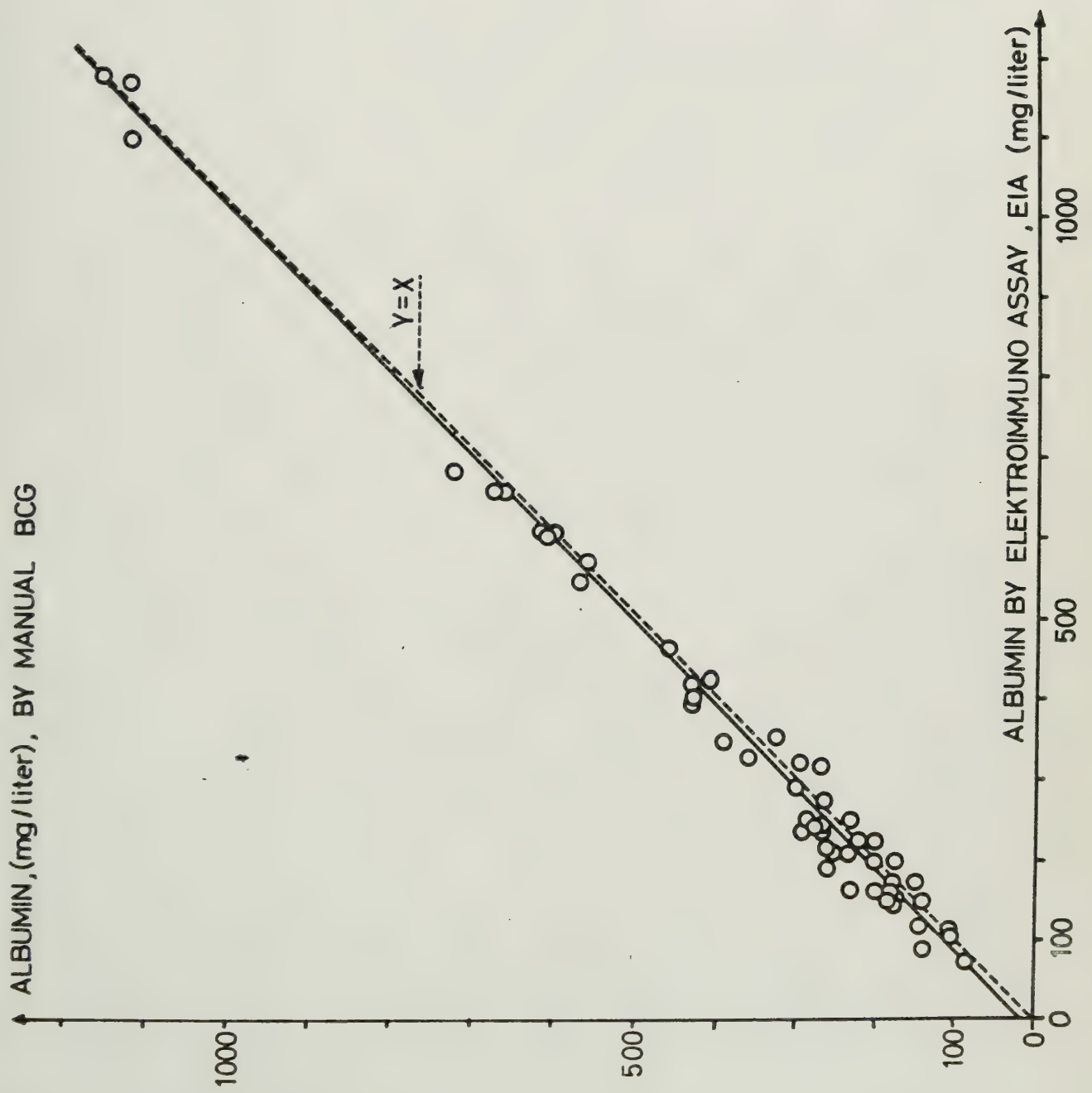
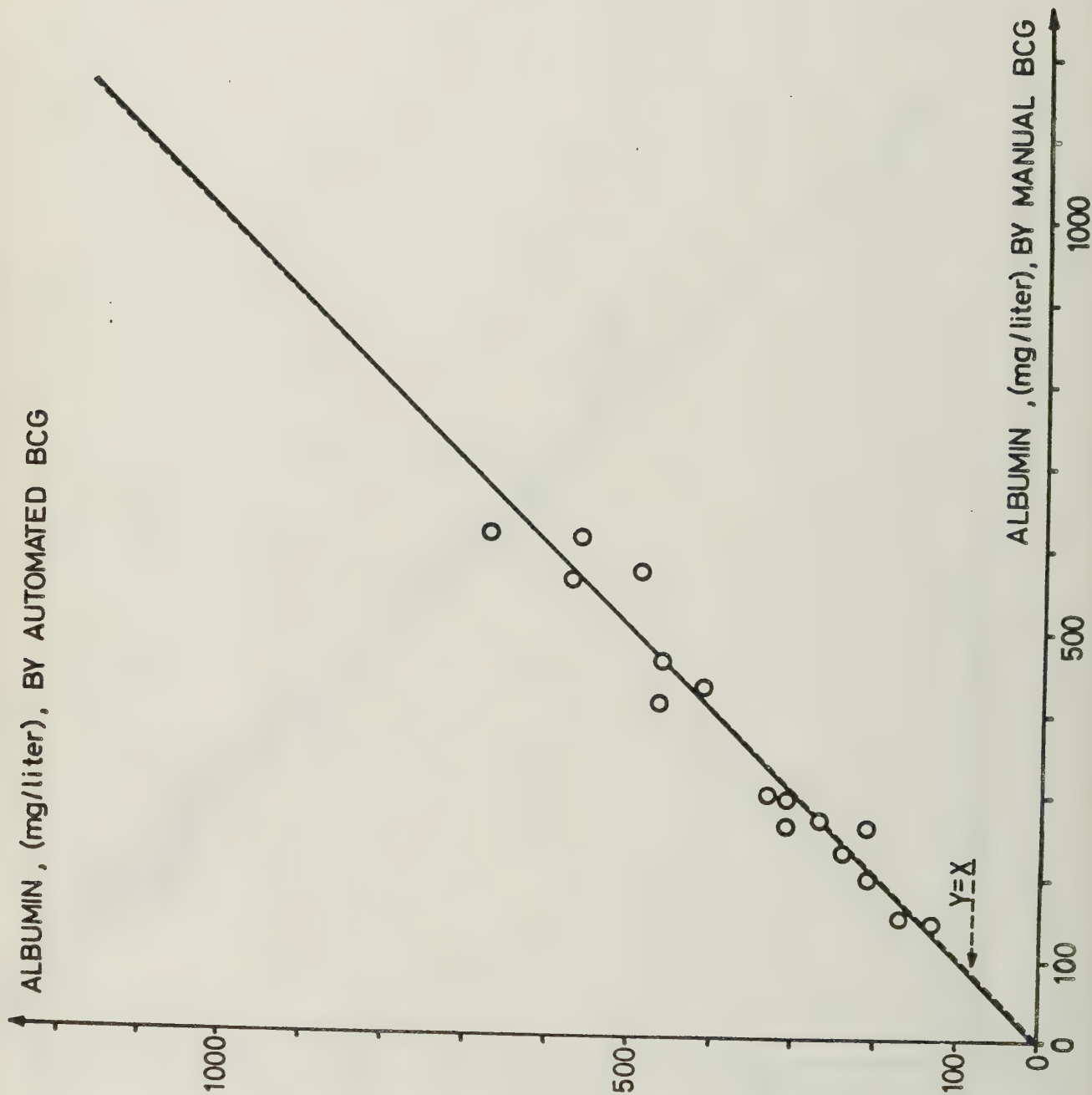


FIGURE 4









Simplified Procedure for Urinary Albumin Determination by the  
Immediate Bromocresol Green Method

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### Summary

It has earlier been demonstrated that the results obtained with the immediate bromcresol green (BCG) method are reliable for determination of albumin in urine. The results compare well with Laurell "rocket" technique and give a good precision.

In this paper a simplified procedure for the pH-adjustment of the urines is described. We used two different indicator dyes, Phenol Red or Bromthymol Blue, which did not interfere with the albumin-BCG-reaction. Either of these can be used.

This simplified method compare well with our earlier published method.

### Key-words

Albumin, Urine, BCG immediate, pH-adjustment, Bromthymol Blue, Phenol Red.

## Introduction

The immediate bromocresol green (BCG) method for urinary albumin determination used here as well as our earlier method (1) is designed to ascertain the albumin concentration for patients with proteinuria. Our intention is to use it together with serum albumin (2) to obtain the albumin clearance. We will also follow the changes of urinary albumin during pregnancy. The purpose was thus not to determine the exact concentration within normal levels of albumin in urine. If the latter is looked for, another dilution factor, eventually with a more concentrated reagent as in (3), can easily be chosen.

This method is a simplified version of the method earlier presented (1). The method can easily be automated i.e. with guidelines given in (3). The immediate BCG method is reliable and accurate for routine use.

## Materials and Methods

### Simplified immediate BCG method

Reagents. The BCG reagent and albumin standard used were those described in (1).

Two different indicator dye solutions were prepared. Phenol Red (PR): Dissolve 0,1 g PR in 28,2 ml 0,01 M NaOH in a 250 ml measuring flask. Add then distilled water to the line

Bromthymol Blue (BTB): Dissolve 0,1 g BTB in 16 ml 0,01 M NaOH in a 250 ml measuring flask. Add then distilled water to the line.

Apparatus. The same as in ref. 2.

Procedure. The same as in ref. 1, except the pH-adjustment procedure for the urines. 3 drops of indicator dye solution are added to each urine sample. For alkaline urines (above  $\sim 6.7$ ), pH-adjustment was made, with 5 mol/l hydrochloric acid, to the acidic colour of the indicator. PR gives a colour change from red to yellow, while BTB changes from blue to yellow. Which indicator dye to be used depends on personal preference and/or on the initial colour of the urine. If the urine contains hemolyzed red blood cells BTB gives a better end point, and if the urine initially is bluish green PR is preferred.

The method thus consists of following steps:

1. Check and adjust the pH of the urines as above.
2. Centrifuge the urine samples.
3. Check approximate albumin content with Albustix
4. Dilute if necessary ( $>4000$  mg/l).
5. Pipet 2.0 ml BCG reagent into the measuring cuvettes.
6. Add 200  $\mu$ l urine sample, mix and read absorbance (629 nm) within a few seconds. Extrapolate back to the time of mixing.
7. Read blank samples.
8. Calculate the albumin concentration in the urine samples, by using a standard curve.

This simplified procedure is compared with the procedure described in (1).

## Results

### Light absorbance at different pH for PR and BTB

Fig. 1 and Fig. 2 show the light absorbance curves at different pH for PR and BTB. It can be seen that at pH 4.20, neither PR nor BTB give any significant absorbance.

### Effect of pH adjustment

The pH-adjustment of the urine samples using either PR or BTB was performed and the final pH was checked with a pH meter using a glass electrode. The results can be seen in Table 1.

### The simplified BCG method compared with earlier BCG method (1)

Both the simplified BCG method using PR and using BTB have been compared with the earlier described BCG method (1). Results are presented in Fig. 3 and 4.

The linear regression curves and correlation coefficients ( $r$ ) calculated by the Deming's procedure as described by Wakkers et al. (4) were:

$$y_1 = 1.5 + 1.002x \quad (n=35; r=0.999)$$

$$y_2 = 8.2 + 0.999x \quad (n=35; r=0.999)$$

$x$  = Earlier BCG method (1)

$y_1$  = BCG method using PR

$y_2$  = BCG method using BTB



### Discussion

A simplified procedure for using the immediate reaction for determination of urinary albumin (1) is described. PR or BTB was used as pH indicator, when adjusting the urinary pH. It was shown that PR and BTB did not interfere with the albumin-BCG reaction (Fig. 3 & 4) and did not have any absorbance at 629 nm, when the pH was 4.2.

Since a strong hydrochloric acid (5 mol/l) was used for pH adjustment and since the urines have very different buffer capacity (1), the final pH varies (Table 1). Nevertheless, the results of the described BCG method for urinary albumin determination were not affected by these pH differences.

The BCG method described compares well with an earlier presented (1) BCG method, both when using PR or BTB. All other findings of the earlier method, including comparison with the Laurell "rocket" technique, are thus true, also for this simplified method. A check of the precision, showed that it is very similar to the earlier method.

If a high throughput of samples is needed, automation of this method can easily be carried out along the guidelines given in ref. 3 by merely changing the dilution factor.

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Table 1.

pH in patient urines after pH adjustment. A pH meter with glass electrode was used.

(PR = Phenol Red, BTB = Bromthymol Blue)

| pH                             |                         |                          |
|--------------------------------|-------------------------|--------------------------|
| Initial pH in<br>urine samples | Adjusted by<br>using PR | Adjusted by<br>using BTB |
| 8.0                            | 6.0                     | 5.8                      |
| 6.8                            | 4.5                     | 4.5                      |
| 8.5                            | 6.2                     | 6.5                      |
| 8.2                            | 4.9                     | 5.1                      |
| 9.0                            | 6.8                     | 6.5                      |
| 8.0                            | 6.3                     | 6.3                      |
| 8.0                            | 4.7                     | 5.7                      |
| 7.0                            | 6.0                     | 5.8                      |
| 7.0                            | 5.8                     | 5.5                      |
| 6.0                            | 5.5                     | 5.4                      |
| 6.5                            | 5.5                     | 5.4                      |
| 6.5                            | 5.3                     | 5.2                      |
| 7.0                            | 6.0                     | 5.4                      |
| 9.0                            | 6.2                     | 6.0                      |
| 7.0                            | 5.5                     | 5.0                      |
| 7.0                            | 4.8                     | 5.5                      |
| 6.8                            | 5.8                     | 4.7                      |

Figure Legends

Fig. 1. Spectral curves at different pH for Phenol Red.

Fig. 2. Spectral curves at different pH for Bromthymol Blue.

Fig. 3. Comparison of urinary albumin results as determined by the immediate BCG method using Phenol Red (y) and the immediate BCG method (1) using Duotest (x). Linear regression analysis, according to Deming's procedure as described by Wakkers et al. (4) was:

$$y = 1.5 + 1.002x \quad (n=35; r=0.999).$$

Fig. 4. Comparison of urinary albumin results as determined by the immediate BCG method using Bromthymol Blue and the immediate BCG method (1) using Duotest (x). Linear regression analysis, according to Deming's procedure as described by Wakkers et al. (4) was:

$$y = 8.2 + 0.999x \quad (n=35; r=0.999).$$



FIGURE 1

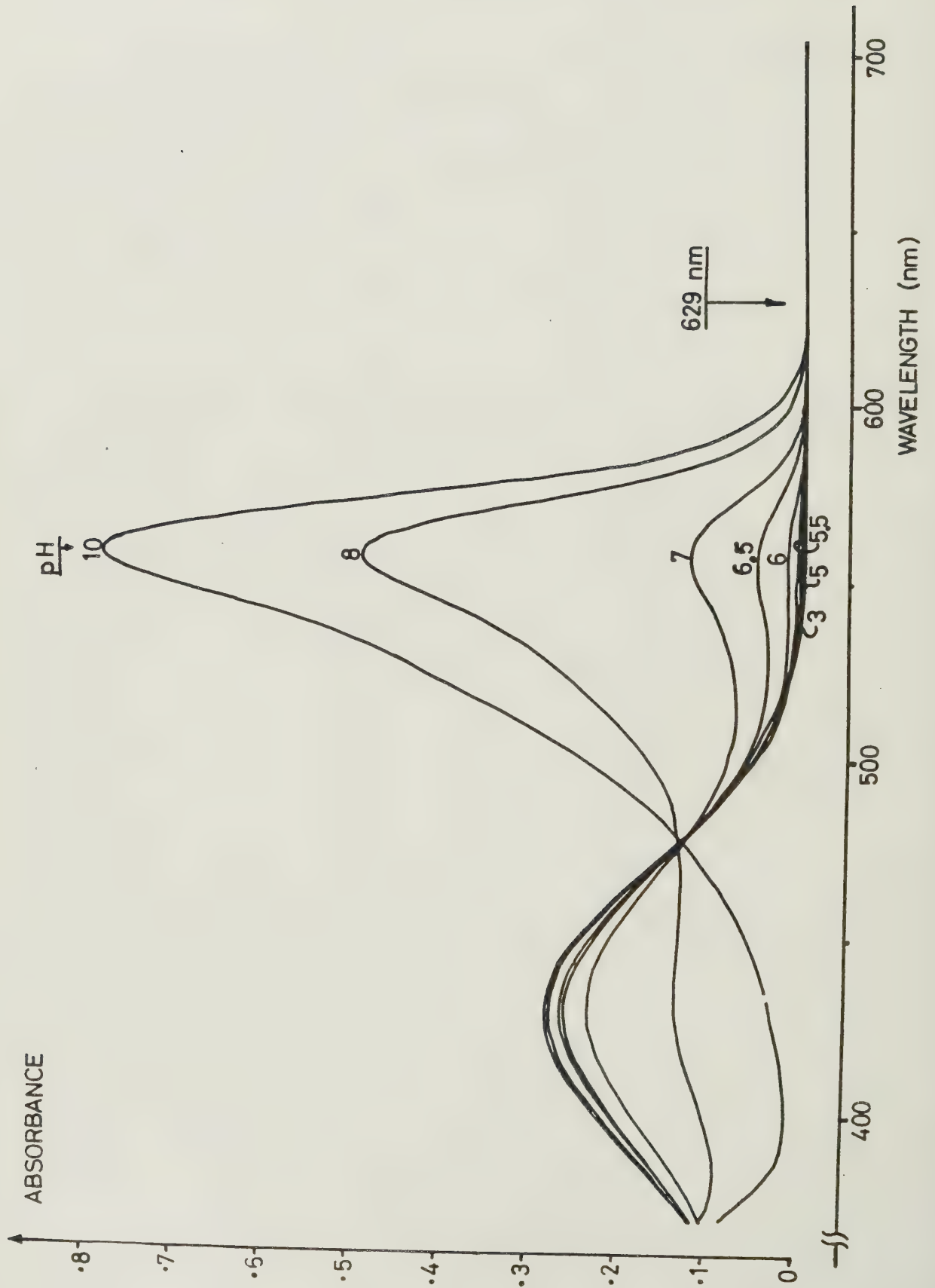


FIGURE 2

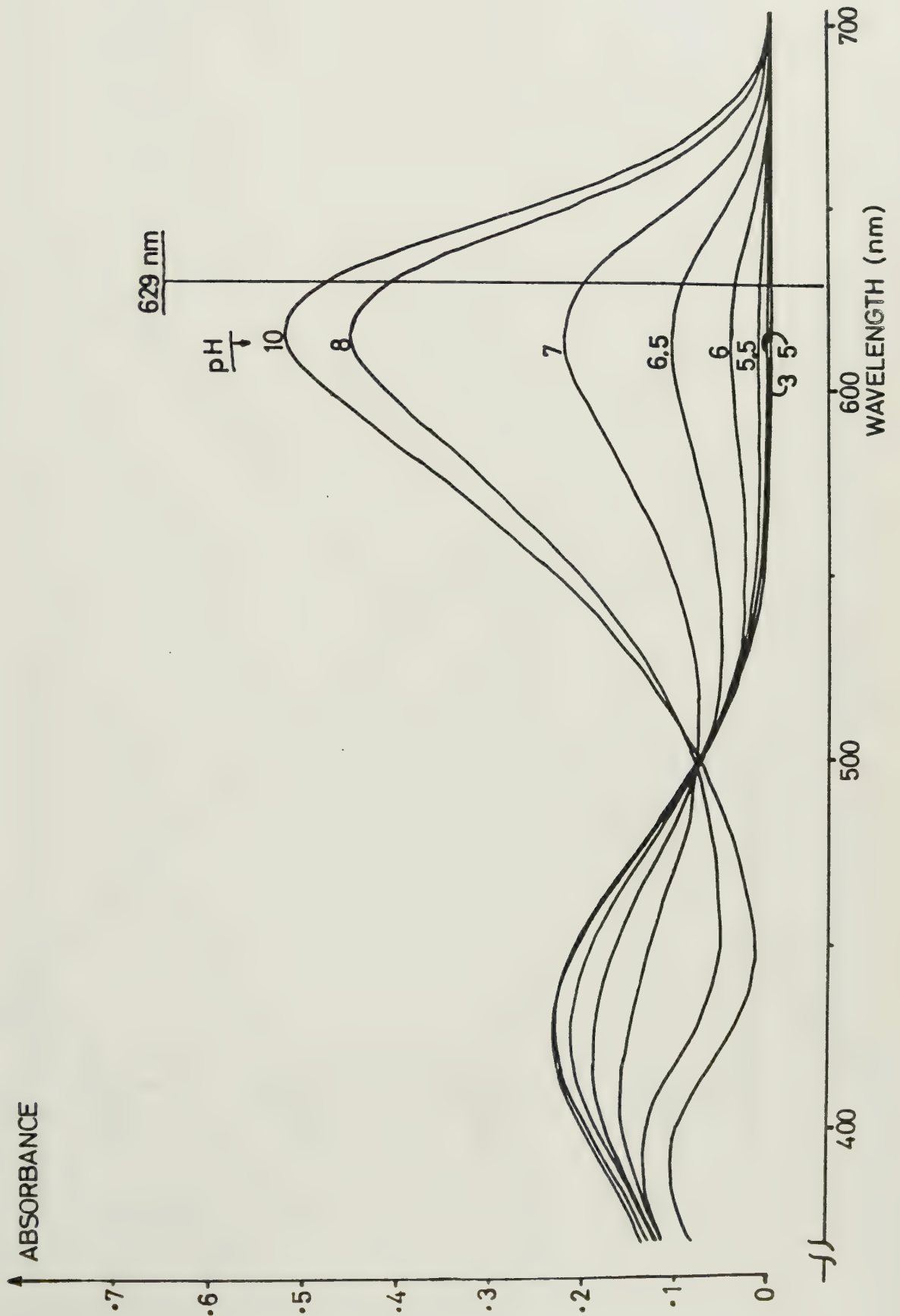


FIGURE 3

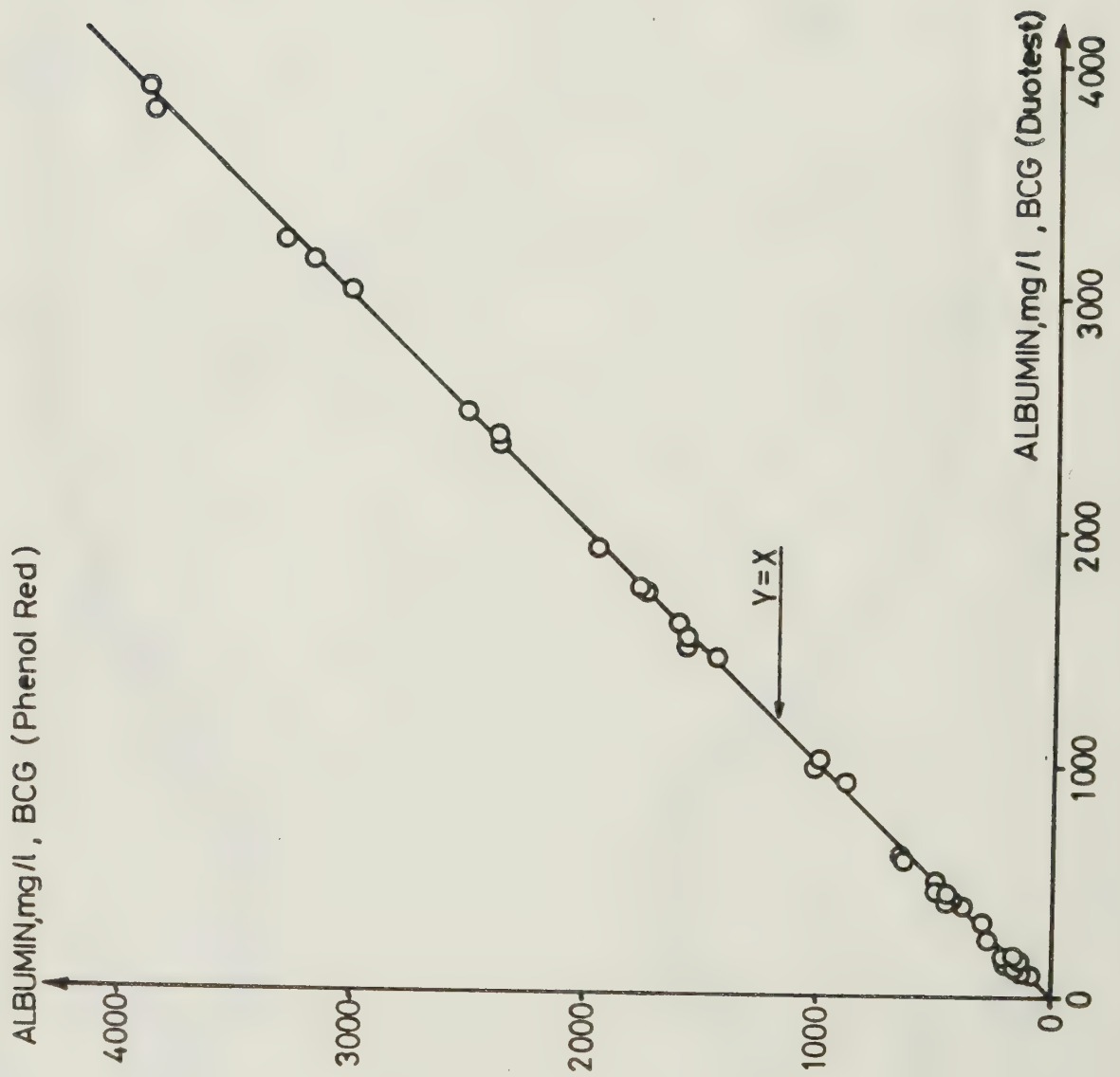
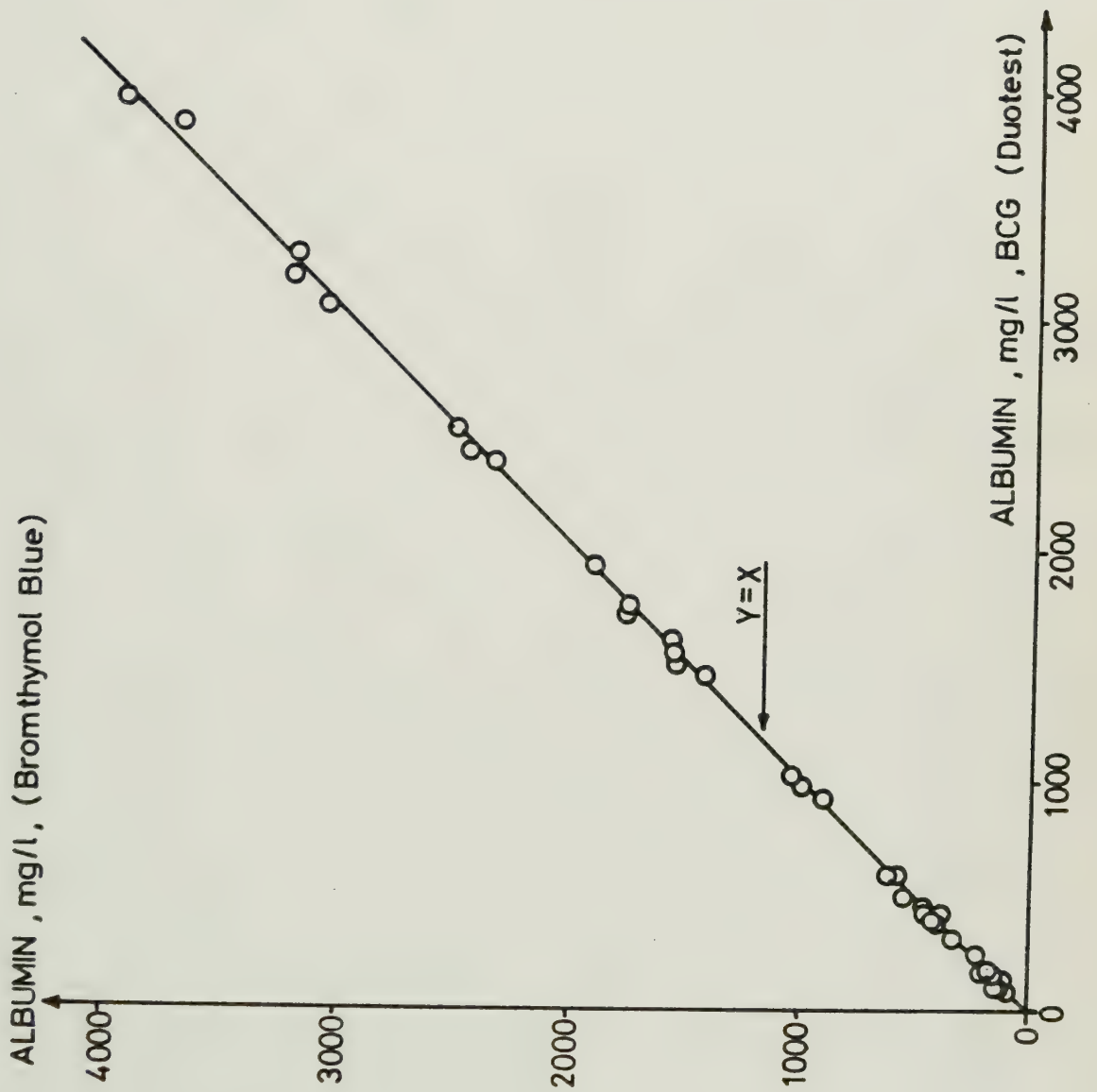


FIGURE 4











Identification of Haptoglobin, Transferrin and  $\alpha_1$ -Antichymotrypsin  
to Cause the Slow Bromcresol Green Reaction with Human Serum,  
and further Demonstration of the Specificity for Albumin of the  
Immediate Reaction.

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Abstract

The reaction of serum samples with bromcresol green proceeds in two steps. It has earlier been shown that albumin is responsible for the immediate reaction while the slower reaction is caused by other serum components. The aim of this study is to identify those slow reacting components. This is performed by using several methods including preparative agarose electrophoresis, gel chromatography, affinity chromatography and by comparison of different patient's sera, Cohn fractions and salting-out fractions. Also commercial purified protein preparations were used. Three serum proteins, causing the slow bromcresol green reaction, were identified. They were transferrin, haptoglobin and  $\alpha_1$ -antichymotrypsin. No other of the major serum proteins reacts slowly or rapidly with bromcresol green. The specificity of the immediate reaction for albumin as earlier presented is also further demonstrated.

## Introduction

Many methods have been described for determining albumin in serum (1-24). The advantages of the bromcresol green (BCG) reaction (12-24) are: rapid determinations, simplicity, suitability for automation and low cost. The reagents are easy to prepare and are stable.

The main disadvantage, the uncertain specificity for albumin has been clarified (14). The remedy was to read the absorbance as soon as possible after mixing sample with the reagent. It was thus shown that albumin specifically reacts immediately with the BCG reagent and that a good agreement was obtained with results obtained by other specific, but time consuming immunological and electrophoretic methods. This was true, also for pathological sera in the clinically important low concentration range. These results have later been confirmed by others (15-22). Webster (16) had a fairly constant difference of 3.0 g/l albumin which he postulated to depend on other fast reacting nonalbumin material in the serum. By using the results in (21) it is more probable that this difference depends on the use of saline for the dilution of serum samples. The saline causes some of the slow reacting components to react fast. The same should be true for (22).

In (14) it was also shown that there are nonalbumin components in serum which react slowly with bromcresol green. These correlate closely with the presence of acute phase reactants. This slow reaction is the main cause why previous investigators using longer reaction times did not find the good correlation indicated above.

Too high "albumin" results for patients with high levels of "acute phase reactants" have been reported (23,24), especially disturbing at and often coinciding with low albumin concentrations for patient's sera.

The aim of this study is to identify the slow reacting components of the BCG-serum reaction. <sup>1</sup>✓ Several techniques were used, including preparative agarose electrophoresis, gel chromatography and affinity chromatography. Also a comparison of different patient's sera, Cohn fractions, salting out fractions and commercial purified protein preparations was performed. Three serum proteins, transferrin (Tf), haptoglobin (Hp) and  $\alpha_1$ -antichymotrypsin (Achy), caused a slow reaction with BCG. No other of the major serum proteins reacts with BCG, either slowly or immediately.

It is thus possible with the very simple BCG method to simultaneously obtain both a reliable albumin value and a measurement of "acute phase reactants" in serum. A pair of values which together can be used in several diagnostic situations are thus obtained.

### Materials and Methods

#### BCG Method

Reagents. The reagents for the manual method were those described by Doumas et al. (13), as previously reported (14). As reference a serum pool from healthy blood donors was used. A special attention to the Brij-35 30% solution must be made. The preparation made by Technicon Instrument Corp., Tarrytown, N.Y. 10591, is declared to contain 30% Brij-35, but upon a specific inquiry to

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<sup>1</sup>✓ Some of the results were presented at the 2nd European Congress on Clinical Chemistry in Prague, Czechoslovakia, October 3-8, 1976.

the company, they informed that it only contains about 23-24% Brij-35. This must be corrected when using this method

Apparatus and procedure. As described earlier (14).

#### Electroimmunoassay (EIA)

The procedure described by Laurell (4, 25), with practical arrangements as before (14), was followed

#### Single Radial Immunodiffusion (SRID)

This technique was originally described by Mancini et al. (3), and was performed according to a manual from the manufacturer of the antisera (Dakopatt A/S DK 2900 Hellerup, Denmark).

#### Preparative and Analytical Agarose Gel Electrophoresis

The procedures described by Johansson (26) were followed.

Preparative agarose gel electrophoresis was performed in a 3 mm thick gel. The electrophoresis buffer used was a barbital/sodium barbital buffer 75 mmol/l, pH 8.6, containing 2 mmol calcium lactate per liter. About 0.8 ml serum was mixed with Indubiose A37 Agarose (L'Industrie Biologique Francaise S.A., Gennevilliers, France) at 53°C and filled into a preformed slit 3x150 mm in the gel on a glass plate (205x110 mm). The electrophoresis run was followed by two smaller slits (each 3x5 mm), one on each side of the larger slit, filled with serum mixed with bromphenol blue. The potential gradient in the gel was about 10 V/cm. The electrophoresis was continued until the albumin front had migrated 5 cm.

The localization of the protein bands were determined by covering the gel with a filter paper, which after removal was dried, dyed



in bromphenol blue, washed and dried again according to Johansson (26). Agarose strips were cut out and the proteins were recovered by a freezing and thawing procedure. The fractions were concentrated to about the original serum volume by ultrafiltration, described separately below.

Analytical agarose gel electrophoresis was performed in a 1 mm thick gel. 15 samples could be processed in one run. Buffer, voltage gradient and migration distance as above. The plate was fixed, washed, dried, dyed, washed and dried again according to Johansson (6).

#### Gel Filtration on Sephadex G 200 and Sepharose-CL 6B

Gel filtration was performed in Sephadex G 200 superfine and in Sepharose-CL 6B (Pharmacia Fine Chemicals AB, Box 175, S-75104 Uppsala 1, Sweden) at room temperature each in a column K 16/100 (Pharmacia Fine Chemicals AB), i.d. 16 mm and a gel bed height of 850 mm. The sample volume was 2.5 ml, and the flow rate was 3 ml/h for Sephadex G 200 and 9 ml/h for Sepharose-CL 6B. The buffer was a 0.075 mol/l barbital/Na-barbital buffer pH 8.6 with 3 mmol/l sodium azide ( $\text{NaN}_3$ ). This buffer was chosen to make it possible to put an aliquot of the fractions from the eluate in the sample wells used in the immunological methods without further dilution or any change of buffer by dialysis. The elution patterns for the serum proteins were followed by an UV-Absorptiometer (LKB Uvicord, LKB-Produkter AB, S-16125 Bromma 1, Sweden) and a recorder (LKB 6520 Chopper Bar Recorder, LKB-Produkter AB). The fractions were collected in the different tubes by a LKB 7000 A

Ultrac fraction collector equipped with a drop counter.

Proteins in the different fractions, each about 1 ml, were quantitated by SRID and EIA as described above and by the BCG method.

#### Cohn Fractions

The Cohn fractions II, III, IV, IV<sub>1</sub> and VI were obtained from Miles Laboratories, Inc., Elkhart, Indiana 46514. The lot numbers were:

II (40), III (19), IV (26), IV-1 (13) and VI (11). Solutions containing the freeze-dried material were prepared and were analyzed

- by the BCG-method and the Biuret method.

In the specifications from the manufacturer, the following is given.

Fraction II: Gamma globulin as usually obtained is 95-96% pure, but Pentex<sup>R</sup> fraction II has been subjected to further purification by the precipitation of contaminants, largely beta globulins, with an acridine derivative. The resulting product is greater than 98% gamma globulin when characterized by cellulose acetate electrophoresis.

Fraction III: The basic fraction III is obtained upon solubilization of the II+III alcohol paste and reprecipitation of the fraction III under the original precipitation conditions. Pentex<sup>R</sup> fraction III contains 70% beta globulins; the principal proteins in this fraction include beta lipoproteins, isoagglutinins, prothrombin and plasminogen as well as C<sub>1</sub>-protein.

Fraction IV contains 50-60% alpha globulins; the principal contaminants are beta globulins and albumin. Fraction IV can be further fractionated into:

fraction IV-1 containing the alpha-1 lipoproteins, ceruloplasmin and various enzyme constituents and fraction IV-4 containing beta-1 metal binding protein, thyroxine binding protein, growth stimulating factors and various enzymes.

Fraction VI. The supernatant resulting from the precipitation of fraction V can be precipitated with heavy metal ions such as barium or zinc, which are subsequently removed by dialysis or diafiltration to yield the acid glycoproteins.

Cohn fraction IV<sub>b</sub> from Kabi AB was also dissolved and subjected to analysis.

#### Affinity Chromatography

Antibodies (10 mg protein/g gel) were coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals AB) by the manufacturer's standard procedure. The same final buffer as in the gel filtration runs was used when preparing the gel.

The antibody containing gels were transferred to columns, K9/15 (Pharmacia Fine Chemicals AB) id 9 mm and gel bed height 130 mm. The flow rate was 20 ml/h. 3 ml serum from a pool of healthy blood donors was applied to the columns, and the columns were washed by buffer until the recorder reached the buffer base line again.

The coupled protein was eluted with a chaotrop ion. In this case a 3.5 mol/liter potassium isothiocyanate was used. The sampling of fractions and the elution pattern was followed with the same equipment as described above for gel filtration.

The fractions were concentrated and the chaotrop ion was removed as described separately below.

The following antibodies were used in the affinity chromatography runs: anti-transferrin, anti-haptoglobin, anti-alpha-2-macroglobulin, anti-ceruloplasmin and anti-haemopexin (All from Dakopatt A/S, DK 2900 Hellerup, Denmark).

#### Salting-out Experiments

A salting-out procedure using ammonium sulphate according to Weeke (27), was performed. The different precipitates were redissolved and were then concentrated and desalted by ultrafiltration in collodium bags as described below. Those solutions were then analyzed by the BCG-method.

#### Concentration of Diluted Protein Eluates

Fractions from the preparative agarose electrophoresis, affinity chromatography and salting-out procedures described above, were concentrated by using collodium bags (Sartorius Membranfilter). The buffer was also changed by repetitively diluting with the preferred buffer and subsequent concentration of the samples in the collodium bags under vacuum.

#### Phenotyping of Haptoglobin

The polyacrylamide gel electrophoresis was in general performed as described in the LKB Application Note No 306. The gel was prepared and moulded in a set, consisting of a slot former, gasket and glass plates (125x260 mm). For the specific procedure for phenotyping of haptoglobin the following procedure was followed. A hemoglobin solution was prepared and mixed with the serum samples before the electrophoresis. A 7.5% polyacrylamide gel concentration in a tris-glycine-buffer, pH 8.9, was used. After



electrophoresis the plate was stained in a benzidine barium peroxide solution. For better visibility and for a permanent appearance the plate was then immersed in a naphtol yellow solution. The blue-green fractions then turned to black. This procedure will be published separately.

#### Commercial Purified Protein Preparations

Two commercial purified protein preparations were used. They were lyophilized powders of transferrin and haptoglobin (Kabi AB, S-10425 Stockholm, Sweden). These powders were dissolved in water (not saline solution!) and the solutions were analyzed by the BCG method and by Mancini radial immunodiffusion for these proteins.

#### Results

##### Estimation of Fast and Slow Reacting Components

The contribution to the absorbance from the fast reaction was estimated by extrapolating the recorder curve to the time of mixing, 0 min. This value was used to calculate the albumin concentration in the samples by comparing with standards with known albumin content run in the same way.

The absorbance difference between 0 and 60 min was calculated and was called  $\Delta A$ . The content in the samples of the "slow reacting components" was then calculated as the difference between "albumin" concentration in g/l at 60 min and at 0 min. This difference is here called  $\Delta C$ .

$$\Delta C = \frac{A_{60} - A_0}{A_{St}} \times C_{St}$$

- $\Delta C$  = "conc." of the slow reacting component  
 $A_{60}$  = absorbance at 60 min reaction time  
 $A_0$  = absorbance at 0 min reaction time  
 $A_{St}$  = absorbance for the albumin standard solution  
 $C_{St}$  = concentration of the albumin standard solution.

#### Preparative Agarose Gel Electrophoresis

Figure 1 shows the analytical agarose gel electrophoresis on the fractions obtained from the preparative agarose gel electrophoresis. Two different types of serum were run, one normal and one with high levels of "acute phase reactants". The BCG method was used for determining the  $\Delta C$  for the different fractions. It is shown that the slow reacting components migrate among the alpha-2- and beta-globulins (Table 1). Some may migrate in the albumin- $\alpha_1$ -region.

#### Gel Filtration on Sephadex G 200 and Sepharose-CL 6B

Figure 2 shows the elution pattern on the Sephadex G 200 column for a serum with high levels of "acute phase reactants" and Figure 3 for a serum obtained from a newborn (cord blood). In addition a commercial lyophilized transferrin preparation (Kabi AB, S-10425 Stockholm, Sweden) was dissolved and chromatographed on this column (Figure 4).

Figure 5-7 show the elution pattern for the three different hapto-globin phenotypes (1-1, 2-1, 2-2) on the Sepharose-CL 6B column. For all chromatographic runs above, all individual fractions, each about 1 ml, were analyzed by the BCG method for albumin and  $\Delta C$

and by SRID or EIA for the specific protein determinations. Several serum proteins were determined. These proteins were compared with the elution pattern for the fast and slow reacting components in the BCG reaction.

It can be seen that the fast reacting component in the BCG reaction compares well with the albumin determined by the immunological methods.

The slow reacting components,  $\Delta C$ , as determined by the BCG method, show two separate maxima. The maximum at the high molecular weight range compares well with haptoglobin in the Sephadex G 200 runs. It is absent in the G 200 run of the serum from a newborn (cord blood), neither could haptoglobin be demonstrated in this serum with immunological technique. The three Sepharose-CL 6B runs with the three different haptoglobin phenotypes show that the position of both the  $\Delta C$  and the haptoglobin peak changes according to the different molecular weights.

The maximum at the low molecular weight range is not fully symmetric. One part of it compares well with transferrin. The comparison of the transferrin and the slow BCG reaction is shown in Figure 4. A full agreement of these curves is shown. In order to identify the second part of this maximum, the difference  $\Delta C$ -haptoglobin-transferrin was also drawn. When comparing this difference curve with the  $\alpha_1$ -antichymotrypsin curve a complete agreement was found. Both curves show two maxima. The molecular weight difference between these two maxima were calculated by using the formula:

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

$K_{av}$  = the fraction of the stationary gel volume, which is available for diffusion of a given solute species.

$V_o$  = void volume

$V_t$  = total volume of the packed bed

$V_e$  = elution volume for a given solute.

Each protein which was analyzed was plotted into a semilogarithmic diagram ( $K_{av}$  vs. molecular weight) to give the selectivity curve for each column. From these selectivity curves the molecular weight difference between the two peaks in the  $\alpha_1$ -antichymotrypsin- and in the difference-curves was estimated to MW about 22000-35000.

#### Cohn Fractions

Six commercially obtainable Cohn fractions were tested. They were No. II, III, IV, IV<sub>-1</sub>, VI and IV<sub>b</sub>. In Table 2 total protein (Biuret) determinations and BCG (fast and slow) can be seen. Fraction IV but not IV<sub>-1</sub> gave a slow reaction with BCG, which means that the slow reacting components are in the IV<sub>-4</sub> fraction. Also the IV<sub>b</sub> fraction reacted slowly with BCG. This fraction is almost identical with IV<sub>-4</sub>. No or only trace amounts of the slow reacting components was found in the other fractions.

#### Affinity Chromatography

The elution pattern was followed by an UV-photometer and a recorder. A typical curve is shown in Figure 8. 3 ml serum was put into the column and eluted with the buffer. When a stable base line was achieved, the bound protein was eluted with a chaotropic ion (3.5 mol/liter KSCN). Two fractions were selected, each containing one of the



peaks as indicated in Figure 8. The two fractions were then concentrated and subjected to desalting and buffer change by ultrafiltration in collodium bags. The volume was then adjusted to the original serum volume (3 ml).

The results from five columns, containing different antisera, are shown in Table 3. Also, the original serum sample was analyzed. It was found that both haptoglobin and transferrin had a slow reaction with BCG, while hemopexin, alpha-2-macroglobulin and ceruloplasmin had not.

#### Salting-out Experiments

Table 4 shows the albumin- and  $\Delta C$ -values by the BCG method and haptoglobin by the hemoglobin binding method, described by Tarukoski (28) on the different salting-out fractions of a normal serum. The components which react slowly with BCG were found in three fractions.

#### Commercial Purified Protein Preparations

The results from the analysis of the water solutions of commercial transferrin and haptoglobin can be seen in Table 5.

#### Discussion

It is already (14) shown, that the BCG reaction with serum samples proceeds in two steps, one immediate and one slow. It was also shown that the measure of the fast reaction compares well with immunological and electrophoresis methods and that reliable albumin results were obtained also in the clinically important low concentration range. The gel filtration experiments described here

(Figure 2-7) show an excellent agreement between the fast reacting component and immunologically obtained albumin results with no extra fractions. This fact together with the results of different kinds of sera (14) and combined with the fact that the albumin fraction from preparative agarose gel electrophoresis reacts fast with BCG without any slow reaction, shows the specificity of the fast BCG reaction for albumin determinations.

The intentions of this paper was also to identify the serum components which react slowly with BCG. Webster et al. (23) compared the BCG method with cellulose acetate electrophoresis giving the typical overestimation of patient's sera with low albumin levels. Webster (24) tried to explain this overestimation by performing the BCG method on fractions from preparative cellulose acetate electrophoresis. The different globulin fractions were treated as a whole and no attempt was made to identify the actual components within the fractions. The time dependence of the reaction was not considered, why only the total reaction was studied.

The separation of the reaction into two steps makes it easier to systematically find out which components in serum react immediately (albumin) and slowly with BCG.

Three proteins were found to cause the slow BCG reaction: Haptoglobin (Hp), Transferrin (Tf) and  $\alpha_1$ -antichymotrypsin (Achy).

The following describes that these three proteins react slowly with BCG and that no other serum protein, at least with a serum concentration above 0.1 g/liter, shows this slow reaction. The preparative

agarose gel electrophoresis, Cohn fractions and salting out experiments disqualify several proteins, including the lipoproteins, but Hp, Tf and Achy fit into all these results (27,29).

It is shown that the slow reacting high molecular weight component in the G 200 separation of an acute phase serum is Hp, because of the absence of this peak and of Hp for a serum from a newborn (cord blood) and because the position of this peak changes exactly as the Hp peak in the Sepharose 6B separations of the three different Hp phenotypes. Furthermore the affinity chromatography experiments give this conclusion.

It is shown that one of the slow reacting components is Tf because of the good fit to the lower molecular weight peak in the G 200 and Sepharose 6B separations and to the Tf peak. The final conclusion can be made by the G 200 separation of a commercial Tf preparation and by the affinity chromatography experiment.

The third slow reacting component is shown to be Achy because of the very good fit between the Achy curve and the "difference curve" ( $\Delta C$  minus Hp minus Tf) in the G 200 and Sepharose 6B chromatography runs. Both Achy and the "difference curve" show a matching double peak. I used the selectivity curve to estimate which difference in molecular weight the distance between the maxima in the double peak corresponds to. I found a MW of about 22000-35000 g/mol. Since the molecular weight of chymotrypsin is about 27000 (30) the two maxima correspond to Achy with and without bound chymotrypsin

The proportion of the height of the two maxima in the double peak is also matching between Achy and the "difference curve" regardless of that the proportion between the two maxima differs between the sera used.

$\Delta C$  can be used as a measure of "acute phase reactants" as described earlier (14), since the increase of the Hp and Achy concentration in serum for patients with an inflammatory reaction is much more pronounced than the resulting decrease of the Tf concentration (31). The increase of Hp and Achy can reach up to eight times of normal concentration while Tf decreases to about 70% of normal. That means that  $\Delta C$ , calculated with the factors in Table 6, reaches about 16-17 g/l (normal sera 5.1 g/l). This level was reached and exceeded in some pathological sera during my work with this method.

In order to calculate the relation between  $\Delta C$  and the protein concentration, both expressed as mol/liter, the following equation is used.

$$\frac{\Delta C}{MW_{alb}} \triangleq \frac{C_{prot}}{MW_{prot}} ; \quad \frac{\Delta C}{C_{prot}} \triangleq \frac{MW_{alb}}{MW_{prot}}$$

$MW_{alb}$  = the molecular weight of albumin.

$C_{prot}$  = concentration of Tf, Hp or Achy.

$MW_{prot}$  = molecular weight of Tf, Hp or Achy.

By using the molecular weights and the results in Table 5, relations according to Table 6 were calculated. It can be seen that BCG reacts with Tf and Achy at the same molar ratio as with albumin. For Hp



about twice as much BCG will react, indicating that each of the two subunits (either the two  $\alpha$ - or the two  $\beta$ -chains), reacts with BCG as albumin does.

It is thus shown that Hp, Tf and Achy react slowly with BCG. Because of the fitness of these curves with the  $\Delta C$  curve in the gel filtration runs described, no other major serum component or at least not one above 0.1 g/liter reacts slowly (or immediately) with BCG.

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Figure Legends

Figure 1. The analytical agarose gel electrophoresis pattern on the fractions obtained by preparative agarose gel electrophoresis. Two types of sera were run, one normal and one with high levels of "acute phase reactants".

Figure 2. A Sephadex G 200 separation of a serum with high level of "acute phase reactants".

Axes:

Abs. = Absorbance;  $C_o$  = Albumin by immediate BCG;  
Albumin = Albumin by SRID; Prot = All other proteins (All concentrations given in g/liter).

Curves:

Abs. = Absorbance curve

Alb. = Albumin by SRID

$C_o$  = Albumin by immediate BCG

$\Delta C$  = Slow reacting components by BCG

Hp = Haptoglobin

Tf = Transferrin

Achy =  $\alpha_1$ -antichymotrypsin

$\Delta$  =  $\Delta C$ -Hp-Tf (difference curve to be compared with the Achy-curve)

$\alpha_2 M$  =  $\alpha_2$ -Macroglobulin

IgA = Immunoglobulin A

IgG = Immunoglobulin G

C3 = Complement C3 ( $\beta_{1A}$ - $\beta_{1C}$ )

Cp = Ceruloplasmin

$\alpha_1$ AT =  $\alpha_1$ -Antitrypsin

Oroso = Orosomucoid

Hpx = Haemopexin

Prealb = Prealbumin

Figure 3. A Sephadex G 200 separation of a serum from a newborn (cord blood).

(Abbreviations, see Figure 2).

Figure 4. A Sephadex G 200 separation of a commercial transferrin preparation.

(Abbreviations, see Figure 2).

Figure 5. A Sepharose-CL 6B separation on a serum with haptoglobin phenotype 1-1.

(Abbreviations, see Figure 2).

Figure 6. A Sepharose-CL 6B separation on a serum with haptoglobin phenotype 2-1.

(Abbreviations, see Figure 2).

Figure 7. A Sepharose-CL 6B separation on a serum with haptoglobin phenotype 2-2.

(Abbreviations, see Figure 2).

Figure 8. A typical elution pattern for an affinity chromatography run. (The first elution peak is splitted because a high sample volume (3ml serum) is used, which means that no buffer is eluted in the center of the sample (shift of baseline)).

Table 1. The migration of the components, which react slowly with BCG, during preparative agarose gel electrophoresis. The haptoglobin content is shown, in order to illustrate the cut between the  $\alpha_2$ - and  $\beta$ -globulins.

| Sample       | Fraction                           | $\Delta C$<br>(g/l) | Haptoglobin<br>(g/l) |
|--------------|------------------------------------|---------------------|----------------------|
| A $\nabla^1$ | Prealb.                            | 0                   | 0                    |
|              | Alb.                               | 0                   | 0                    |
|              | Alb- $\alpha_1$                    | 0.2                 | 0                    |
|              | ( $\alpha_1$ )- $\alpha_2$         | 2.2                 | 1.7                  |
|              | $\alpha_2$ - $\beta$               | 3.4                 | 1.2                  |
|              | ( $\beta$ )- $\gamma_A$ $\nabla^3$ | 0.4                 | 0                    |
|              | $\gamma_K$ $\nabla^4$              | 0                   | 0                    |
| B $\nabla^2$ | Prealb.                            | 0                   | 0                    |
|              | Alb.                               | 0                   | 0                    |
|              | Alb- $\alpha_1$                    | 0.1                 | 0                    |
|              | ( $\alpha_1$ )- $\alpha_2$         | 1.8                 | 0.7                  |
|              | $\beta$                            | 1.5                 | 0.1                  |
|              | $\gamma_A$                         | 0                   | 0                    |
|              | $\gamma_K$                         | 0                   | 0                    |

$\nabla^1$  A = Serum with high levels of "acute phase reactants"

$\nabla^2$  B = Normal serum (pool from healthy blood donors)

$\nabla^3$  = Immunoglobulins, slit to anode

$\nabla^4$  = Immunoglobulins, slit to cathode



Table 2. Total protein determination and BCG (fast and slow) determination on dissolved Cohn fractions.

| Cohn fraction<br>No. | Total protein<br>by Biuret<br>(g/l) | Albumin ( $C_o$ )<br>by BCG<br>(g/l) | $\Delta C$<br>by BCG<br>(g/l) |
|----------------------|-------------------------------------|--------------------------------------|-------------------------------|
| II                   | 3.4                                 | 0.14                                 | 0                             |
| III                  | 2.1                                 | 0.23                                 | 0.10                          |
| IV                   | 4.6                                 | 1.34                                 | 1.17                          |
| IV-1                 | 1.0                                 | 0.40                                 | 0.06                          |
| VI                   | 2.0                                 | 0.39                                 | 0                             |
| IV <sub>b</sub>      | 4.6                                 | 1.32                                 | 1.25                          |

Table 3. Affinity chromatography results with five different anti-sera coupled, each in a separate column. Each was divided into two fractions, before and after use of a chaotop ion. All fractions were analyzed according to the headings in the table.

| Column                             | Eluate          | Total Protein<br>(g/l) | Albumin<br>by BCG<br>(g/l) | A C<br>by BCG<br>(g/l) | Hapto-<br>globin<br>(g/l) | Trans-<br>ferrin<br>(g/l) | Hemo-<br>pexin<br>(g/l) | Cerulo-<br>plasmin<br>(g/l) | $\alpha_2$ -macro-<br>globulin<br>(g/l) |
|------------------------------------|-----------------|------------------------|----------------------------|------------------------|---------------------------|---------------------------|-------------------------|-----------------------------|-----------------------------------------|
| anti-hapto-<br>globin              | 1 $\frac{2}{3}$ | 70.7                   | 42.57                      | 3.90                   | 0.01                      | 2.28                      | 0.89                    | 0.33                        | 1.79                                    |
|                                    | 2 $\frac{3}{3}$ | 1.66                   | 0.53                       | 0.50                   | 0.47                      | Tr $\frac{2}{3}$          | 0                       | 0                           | Tr                                      |
| anti-trans-<br>ferrin              | 1               | 74.4                   | 45.96                      | 3.56                   | 0.89                      | 0.18                      | 0.79                    | 0.33                        | 1.79                                    |
|                                    | 2               | 1.57                   | 0.63                       | 0.80                   | Tr                        | 0.96                      | 0                       | 0                           | Tr                                      |
| anti-hemo-<br>pexin                | 1               | 78.5                   | 47.42                      | 4.96                   | 0.92                      | 2.55                      | 0.37                    | 0.35                        | 2.00                                    |
|                                    | 2               | 0.64                   | 0.50                       | 0                      | Tr                        | Tr                        | 0 (!)                   | 0                           | Tr                                      |
| anti- $\alpha_2$ -macro-<br>globin | 1               | 79.6                   | 47.09                      | 5.19                   | 0.95                      | 2.58                      | 0.80                    | 0.34                        | 0.26                                    |
|                                    | 2               | 0.64                   | 0.77                       | 0                      | Tr                        | Tr                        | 0                       | 0                           | 0.26                                    |
| anti-cerulo-<br>plasmin            | 1               | 81.2                   | 48.36                      | 5.39                   | 0.96                      | 2.61                      | 0.83                    | 0                           | 1.81                                    |
|                                    | 2               | 0.37                   | 0.37                       | 0                      | Tr                        | Tr                        | 0                       | 0 (!)                       | Tr                                      |
| original serum sample:             |                 | 78.1                   | 45.29                      | 5.19                   | 0.87                      | 2.41                      | 0.80                    | 0.35                        | 1.89                                    |

$\frac{1}{3}$  Tr = Trace amount

$\frac{2}{3}$  before addition of a chaotop ion

$\frac{3}{3}$  after addition of a chaotop ion

Table 4. Salting-out experiments. BCG (fast and slow) and haptoglobin (Tarukoski, (28)) determination of the fractions.

| Ammonium sulphate<br>concentration range<br>(mol/liter) | Albumin<br>by BCG<br>(g/liter) | $\Delta C$<br>by BCG<br>(g/liter) | Haptoglobin<br>by Tarukoski (28)<br>(% of total) |
|---------------------------------------------------------|--------------------------------|-----------------------------------|--------------------------------------------------|
| > 3.15                                                  | 3.15                           | 0                                 | 5                                                |
| 3.15-2.20                                               | 42.25                          | 2.85                              | 40                                               |
| 2.20-1.84                                               | 3.50                           | 0.20                              | 10                                               |
| 1.84-1.49                                               | 2.50                           | 1.45                              | 45                                               |
| 1.49-1.13                                               | 0.95                           | 0                                 | 0                                                |
| < 1.13                                                  | 0.80                           | 0                                 | 0                                                |

Table 5. Water solutions of commercial transferrin (Tf) and haptoglobin (Hp) preparations analyzed by the BCG and Mancini radial immunodiffusion methods.

| Preparation | $\Delta C$<br>by BCG | Tf        | Hp        |
|-------------|----------------------|-----------|-----------|
|             | (g/liter)            | (g/liter) | (g/liter) |
| Transferrin | 8.51                 | 10.65     | -         |
| Haptoglobin | 3.12                 | -         | 2.24      |



Table 6. Calculated molecular ratios and found ratios between the slow BCG reaction and corresponding protein.

| Protein                     | molecular weight(MW)<br>(g/mol) | $\frac{MW_{alb}}{MW_{prot}}$ | $\frac{\Delta C}{C_{prot}}$ | $\frac{\frac{\Delta C}{C_{prot}}}{\frac{MW_{alb}}{MW_{prot}}}$ |
|-----------------------------|---------------------------------|------------------------------|-----------------------------|----------------------------------------------------------------|
| Transferrin                 | 77 000 <sup>1/</sup> ✓          | 0.86                         | 0.80                        | 0.9                                                            |
| Haptoglobin (1-1)           | 100 000 <sup>2/</sup> ✓         | 0.66                         | 1.39                        | 2.1                                                            |
| $\alpha_1$ Antichymotrypsin | 69 000 <sup>1/</sup> ✓          | 0.96                         | 1 <sup>3/</sup> ✓           | 1.0                                                            |
| Albumin                     | 66 000 <sup>1/</sup> ✓          | -                            | -                           | -                                                              |

<sup>1/</sup>✓ Blombäck, B., and Hanson, L.Å., Plasma Proteins, AWE/Gebers, Uddevalla 1976.

<sup>2/</sup>✓ Schultze, H.E., and Heremans, J.F., Molecular Biology of Human Proteins with special Reference to Plasma Proteins, Elsevier publ. Co., Amsterdam, 1966.

<sup>3/</sup>✓ By comparing the Achy curve with the difference ( $\Delta C - C_{Hp} - C_{Tf}$ ) curve in the elution patterns from the gel filtration curves.

FIGURE 1

SL-6172  
24/9-75



FIGURE 2

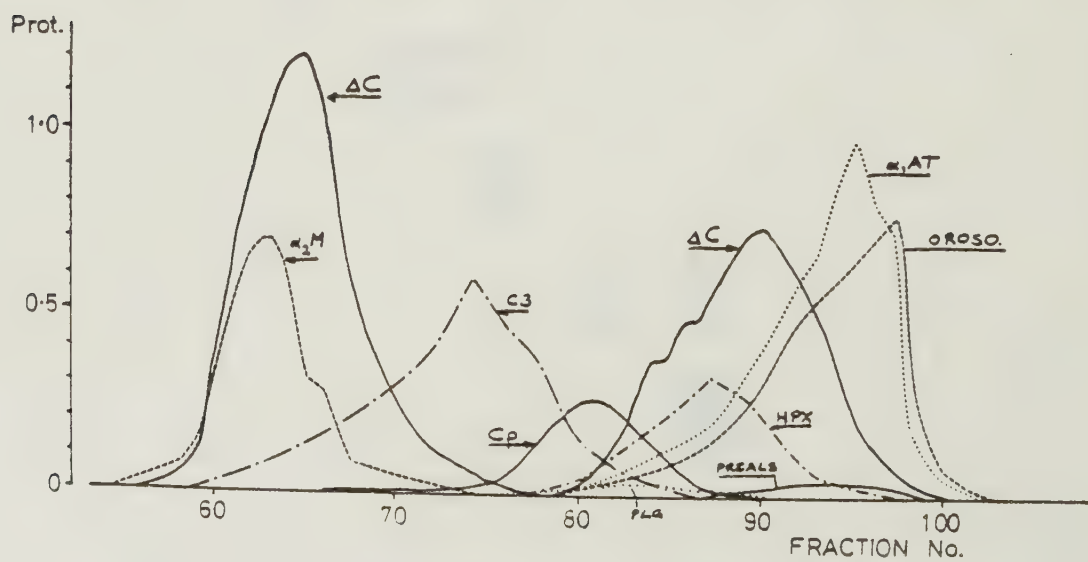
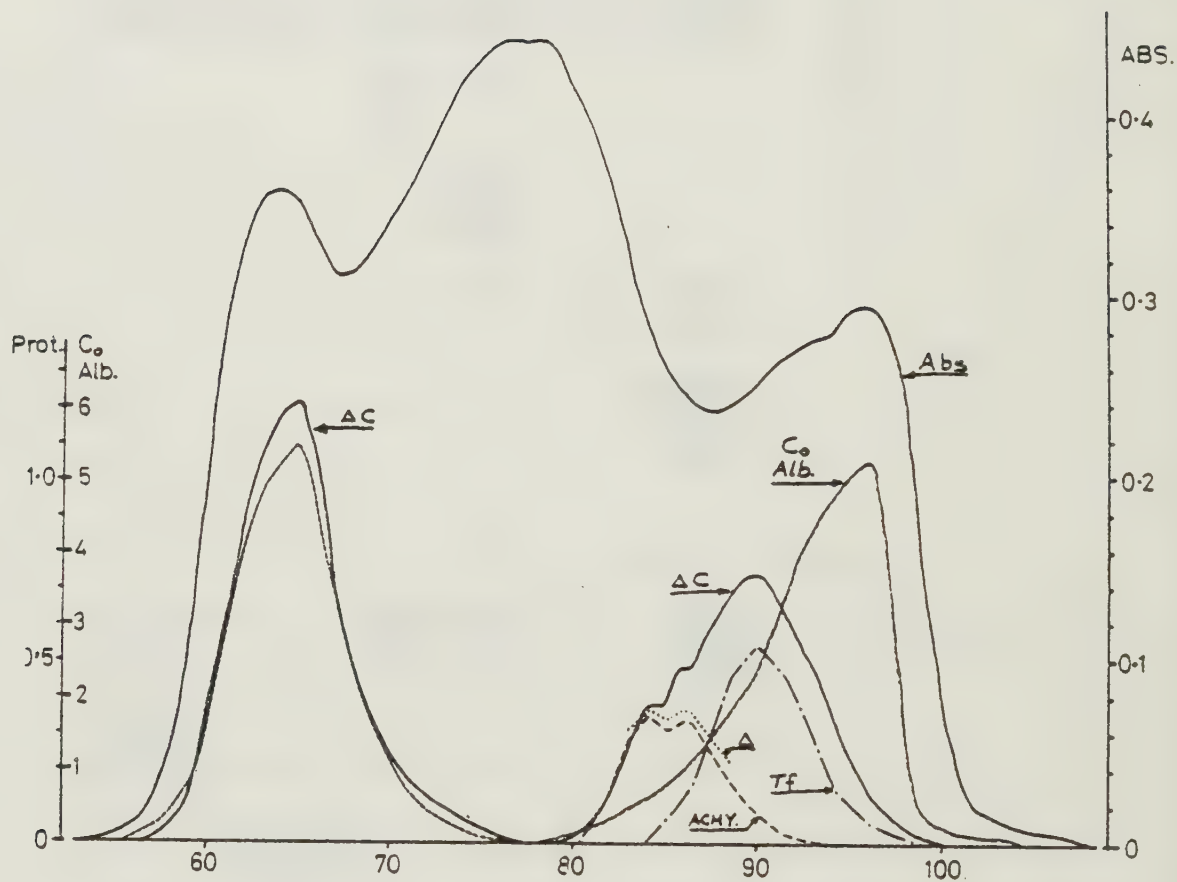


FIGURE 3

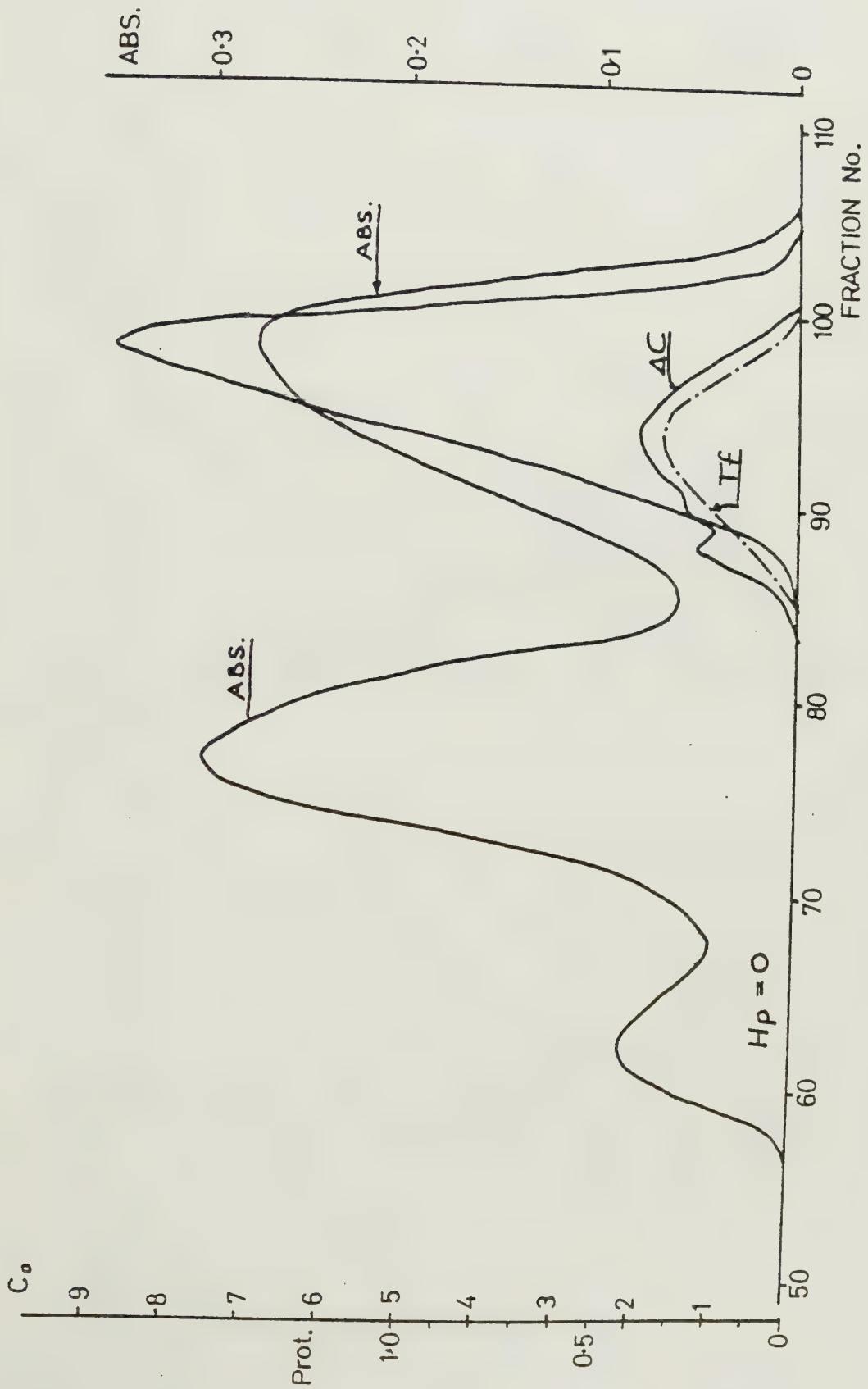




FIGURE 4

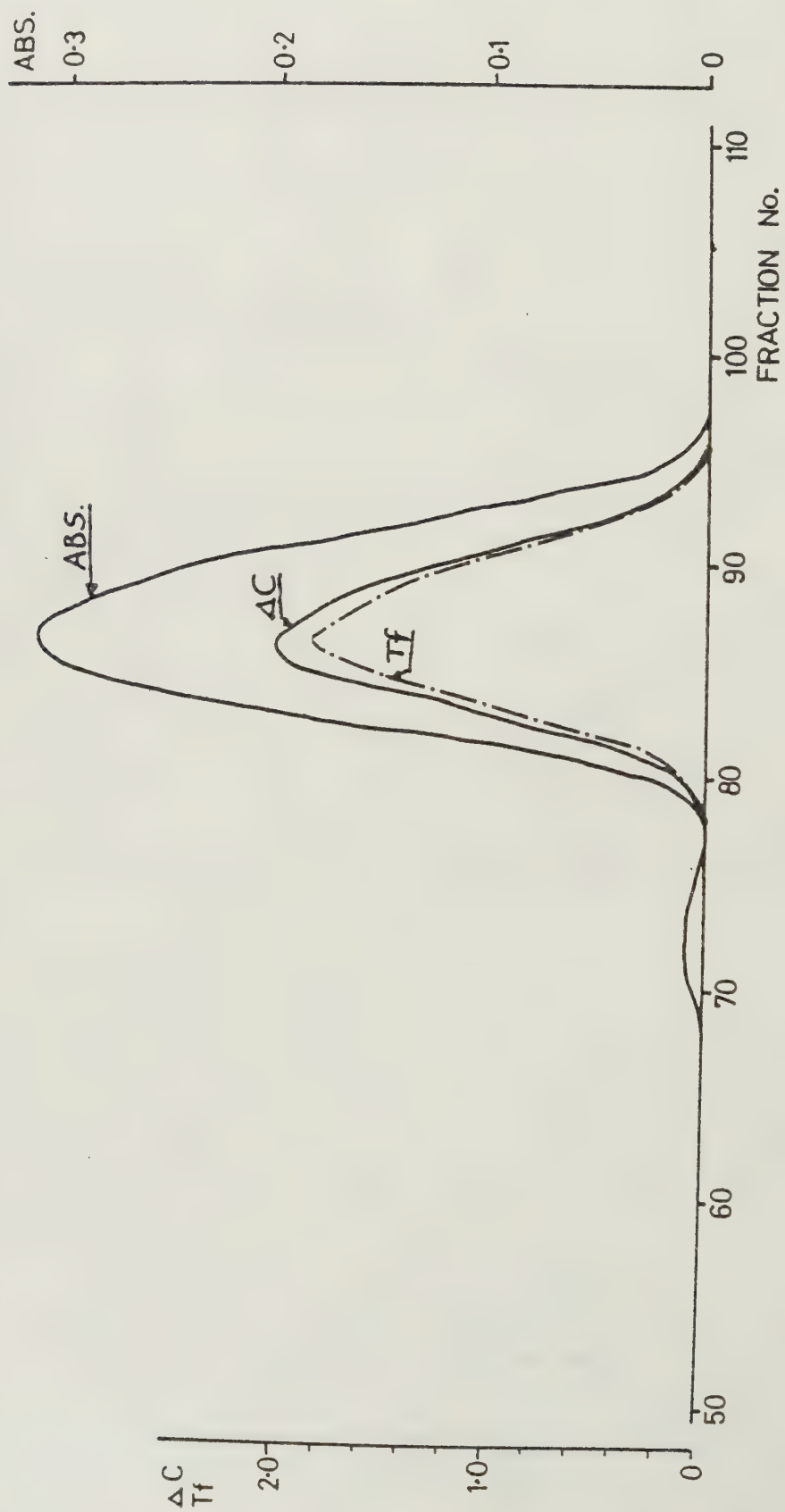


FIGURE 5

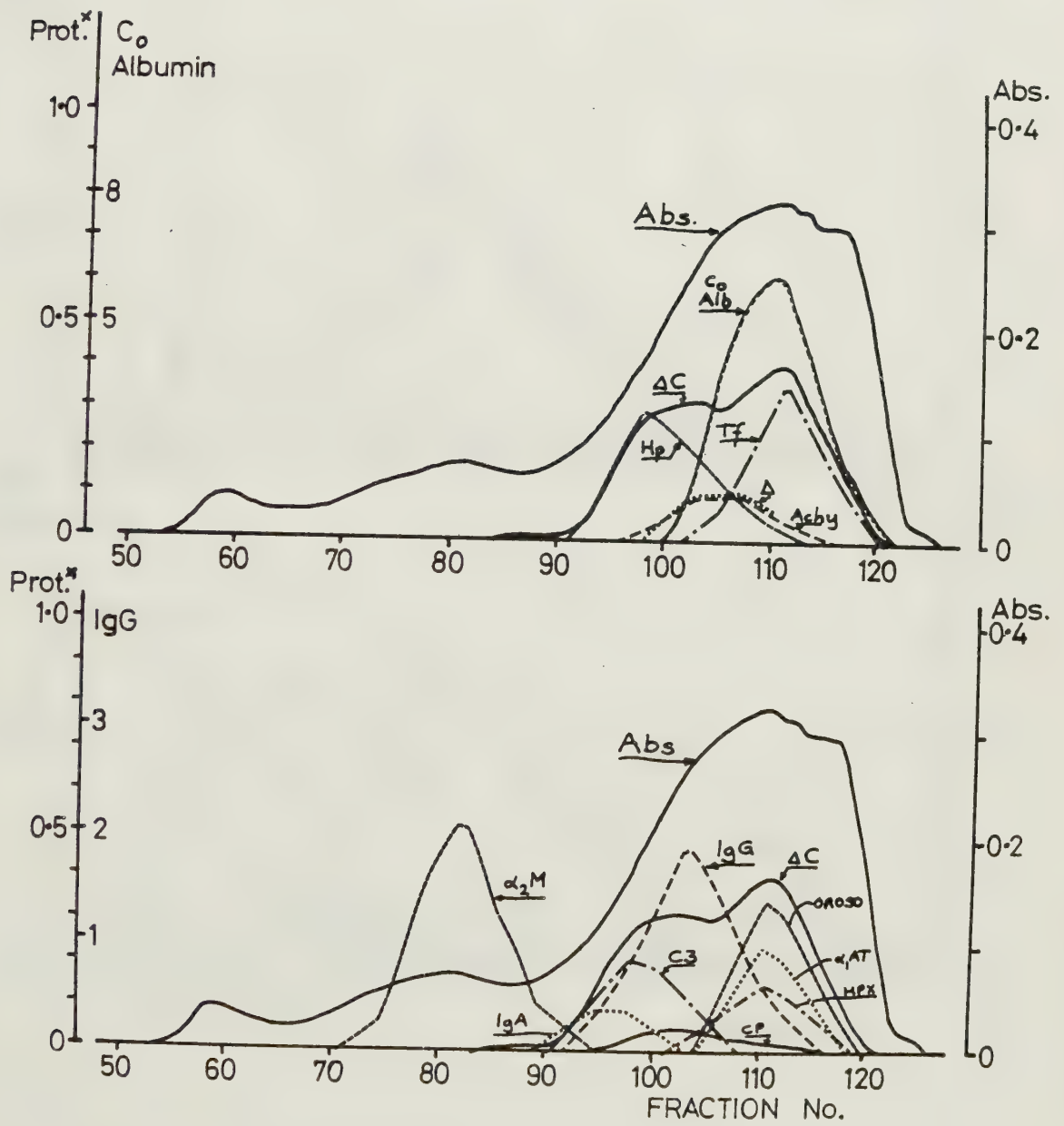


FIGURE 6

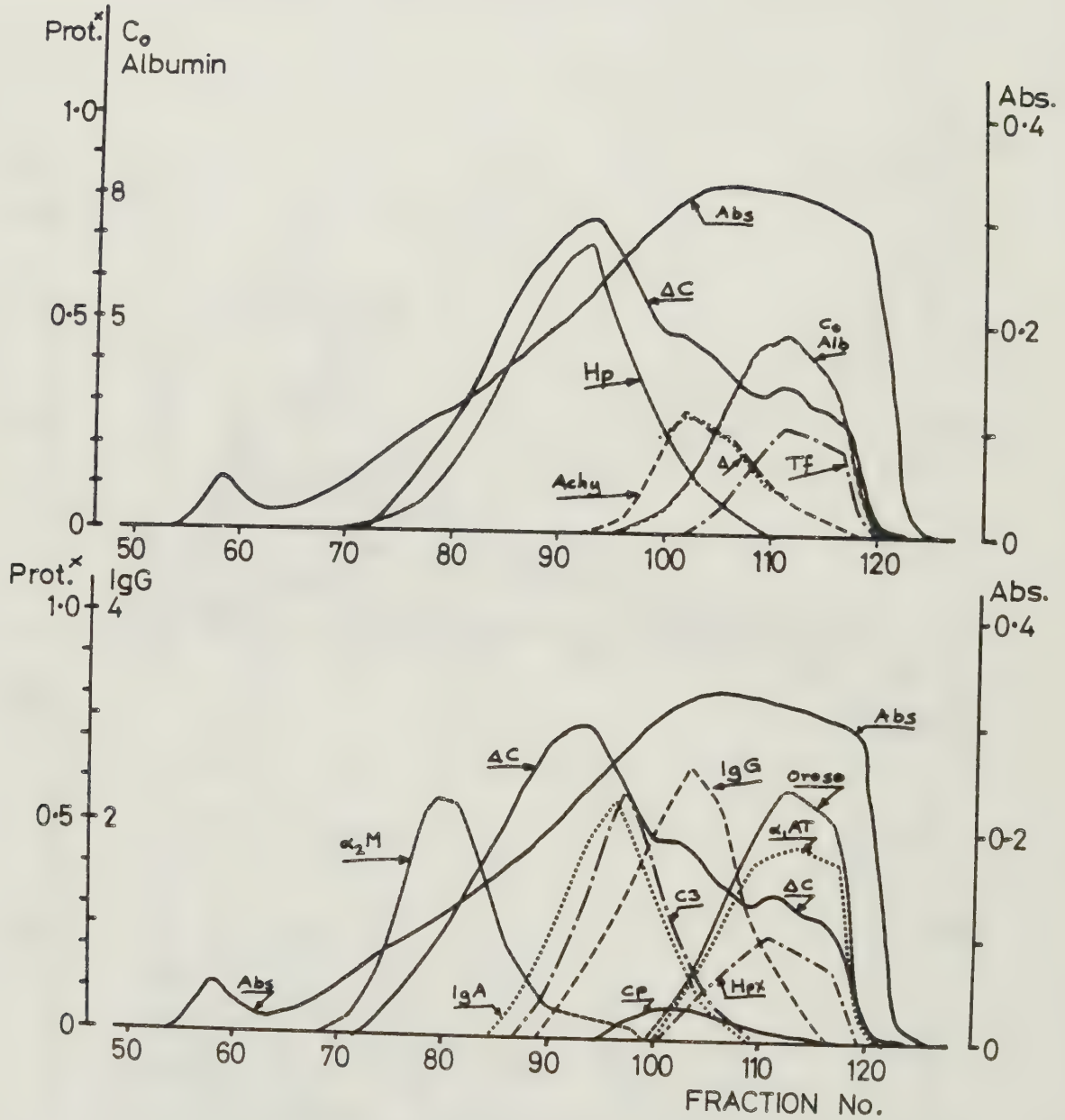


FIGURE 7

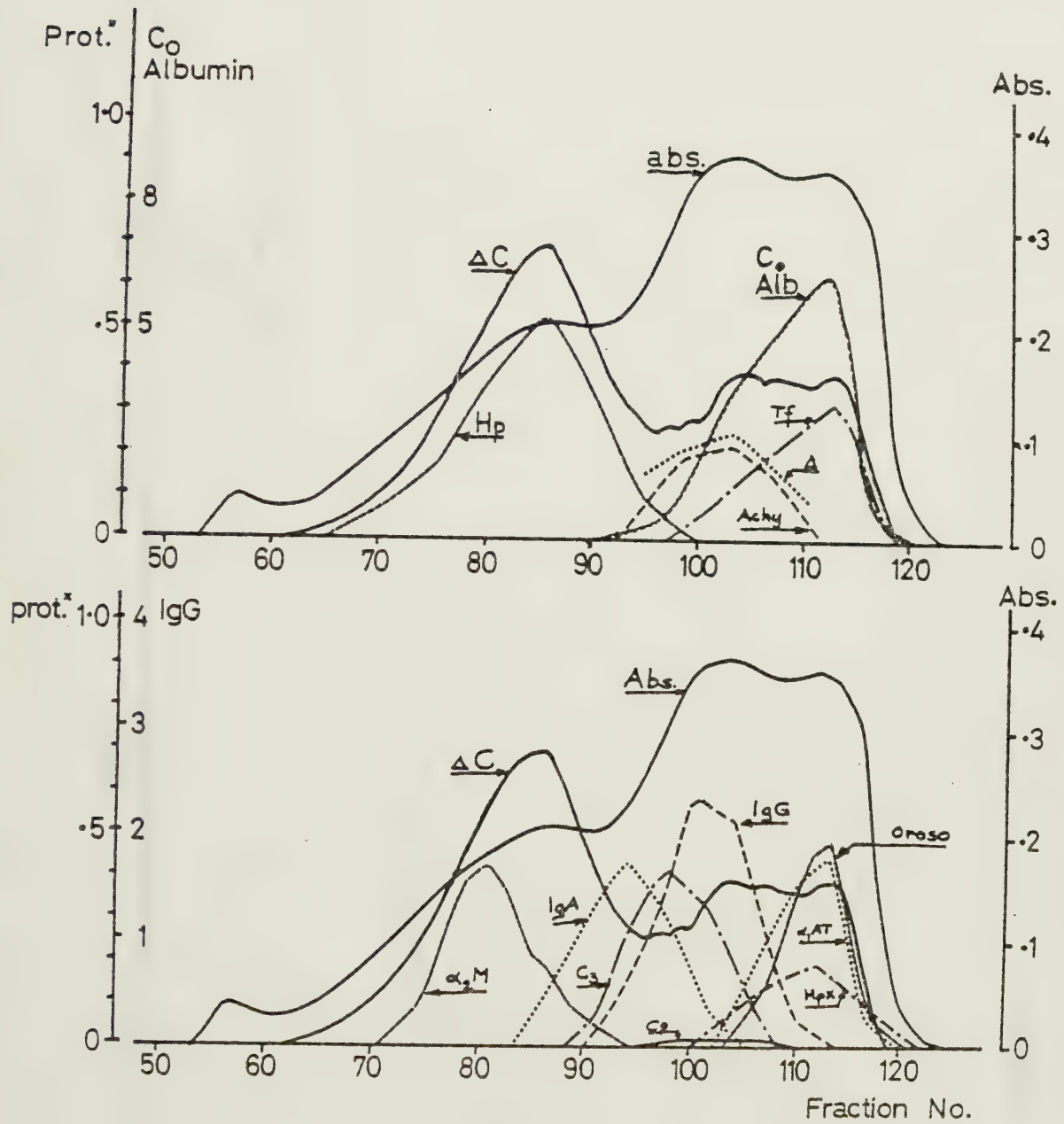




FIGURE 8

